

**HOMOGENEOUS AND HETEROGENEOUS ASSAY METHODS USING
NUCLEATION CENTERS AND NOVEL COVALENT CHEMISTRIES FOR THE
RAPID MEASUREMENT OF PHOSPHORYLATION AND DE-
PHOSPHORYLATION**

FIELD OF THE INVENTION

[0001] The present invention relates to assays for the rapid measurement of phosphorylation and de-phosphorylation and may be used for determining activity of kinases and phosphatases. Also described are combinations of phosphoryl donor substrates, covalent phosphoryl conjugation chemistries and synthetic nucleation centers. Phosphoryl donors described herein may be used to develop homogeneous and heterogeneous assays for monitoring the activity of protein kinases and phosphatases and to enable rapid, quantitative detection of phosphoro, phosphoroamino or phosphorothioate groups on peptide, oligonucleotide or lipid targets of kinase and phosphatase enzymes.

[0002] These substrates, chemistries and assay methods are useful in homogeneous and heterogeneous assays to measure kinase and phosphatase activities *in vitro* and *in vivo*. In other applications, the chemistries described herein can also be used to create molecular tools that can be used to label oligonucleotides, peptides and lipids for use in other biological assay applications.

BACKGROUND OF THE INVENTION

[0003] Protein kinases comprise a major class of therapeutic targets for novel drug discovery and may be classified according to the amino acid residues they phosphorylate. The vast majority of phosphorylation events occur on serine or threonine residues, while a smaller number are directed toward tyrosine residues.

[0004] An analysis of the protein kinase superfamily component of the human genome (the "kinome") recently identified 518 protein kinases and classified them into seven major groups, as well as additional families and subfamilies. See, Manning et al., *Trends in Biochemical Sciences*, 27(10), 514 (2002). Two of these groups are the "AGC" group (containing PKA, PKG and PKC families) and the "TK" group (tyrosine kinase).

[0005] The Protein Kinase C family (PKC) of serine/threonine protein kinases is a group which has been reported to be involved in complex intracellular signaling processes that promote phospholipid hydrolysis. At least eleven isoforms of PKC have been identified. The

isoforms are grouped into three classes (conventional, novel and atypical) based on their structures, activators and dependence on calcium and phospholipid.

[0006] Network formation has been reported to be regulated by, and responsive to, the actions of a wide range of kinase enzymes. For example, actin polymerization and motility are reportedly stimulated by phorbol myristate acetate, a tumor promoter that activates PKC. Brock et al., *J. Cell. Physiol.*, 157, 367 (1993). Actin assembly from soluble subunits has been reported to begin with a thermodynamically unfavorable nucleation step and to proceed efficiently once nucleation occurs. Regulation of actin-membrane associations has been reported to take place through the control of actin assembly *per se* or by modification of linker proteins. Bershadsky et al., *J. Cell. Sci.*, 108, 1183 (1995). Modifications reported include phosphorylation and de-phosphorylation reactions or interactions of the proteins with membrane lipids, particularly phosphoinositides. Stossel, *Nat. Cell Biology*, 1, 75 (2000).

[0007] One property of many plasma-membrane proteins is their association with the underlying cytoskeleton to determine cell shape, and to participate in adhesion, motility and other plasma-membrane processes, including endocytosis and exocytosis. Actin network attachments are said to serve as mechanical linkers between the cell and the substratum or other cells, as locations to assemble cell signal transduction intermediates, as mechanosensors and as foci for the organization of secretory processes. Verhovsky et al., *J. Cell. Sci.*, 110, 1693 (1997). Actin polymerization mechanisms have been noted as a diverse set of examples of the importance of nucleation centers in cell biology.

[0008] Phosphorylation by specific kinase enzymes has been reported to be one of the key mechanisms by which cells transmit information about the activation of a specific receptor to the nucleus, which may eventually result in modifying gene expression. Kim et al., *J. Biol. Chem.*, 274, 9531 (1999). The specific set of kinase enzymes activated in the process are referred to as a "pathway". Activation of a particular pathway is reported to be cell-type specific and generally based upon the actions of a select set of proteins, lipids and ions. Activation of specific pathways may also be dependent upon the specific spatial localization of enzymes in discrete molecular structures within cells, and may occur on the cell membranes. In some cases, activation of a kinase pathway is reported to be initiated by ligand binding at a membrane receptor and to involve ion flux and phosphorylation of the kinase itself. Yang et al., *Mol. Cell. Biol.*, 9, 1227 (2002).

[0009] In contrast, deactivation of a kinase pathway is reported to be controlled by a combination of negative feedback events, which may include proteins that deactivate

receptors, recycling of receptors and phosphatases that de-phosphorylate kinases or their target substrate.

[0010] The kinase and phosphatase enzymes which are said to regulate the phosphorylation of hydroxyl groups on proteins, oligonucleotides and lipids (collectively and individually, the targets or target substrates) are also reported to play roles in a range of signal transduction, cell cycle, gene expression and protein translation pathways. The catalytic activities reported for these enzymes include activities in modifying the extent of phosphorylation of many different biologic targets and pathways, or the activities may be target substrate and signal transduction pathway specific.

[0011] The mechanisms through which phosphorylation and de-phosphorylation of proteins and lipids have been said to accomplish their biological effects have been varied and include conformational changes in specific proteins that then change their biological activities and changes in lipid solubility which subsequently alter the subcellular compartments in which they are active. The kinase and phosphatase enzymes as a group are said to play roles in homeostasis and cellular control.

[0012] Measurement of phosphoryl groups on proteins, lipids or nucleic acids *in vitro* has been reported using assays involving (i) the use of radioactive phosphorous, (ii) the use of non-covalent interactions such as those with polyions, and (iii) the use of anti-phosphoryl antibodies which may be conjugated to extrinsic labels. Enzyme-specific cell based *in vivo* assays have been reported.

[0013] *In vitro* measurements of phosphorylation and de-phosphorylation, as well as phosphodiesterase activities, employing radioactive phosphorous isotopes such as ^{32}P and ^{33}P have been reported.

[0014] Scintillation proximity assays (SPA) are homogeneous assays employing microscopic beads that are impregnated with scintillant molecules that can be stimulated to emit light when radiolabeled molecules of interest are bound to the surfaces of the beads.

[0015] Anti-phosphoryl antibody-based detection methods employ monoclonal antibodies for which the antigenic determinant is a specific phosphorylated peptide or oligonucleotide sequence. In such reported methods, the label is usually attached to the antibody, and, thus, would provide an "indirect" measurement of phosphorylation. A number of antibodies against phosphoryl tyrosines are reported to be obtained; antibodies against phosphorylated serine or threonine sites or specific for phosphorylated oligonucleotide sequences have been less commonly reported.

[0016] One anti-phosphoryl antibody-based detection method was said to involve indirect detection using time resolved fluorescence methods. The assay was said to exploit the long excited state fluorescence lifetime of lanthanide chelates and to utilize one of two types of time resolved detection methods, namely DELFIA (a heterogeneous time-resolved fluorometric method) and LANCE (a homogeneous time-resolved fluorometric method).

[0017] Another anti-phosphoryl antibody-based detection method was said to involve indirect measurements based upon competition assays, where the measurement of phosphorylation was made by a comparison of the competition of fluorescent and non-fluorescent peptide substrates for anti-phosphoryl antibodies. A reported variation of the competitive method involved a heterogeneous colorimetric tyrosine kinase assay which was based upon an ELISA technique where the extent of phosphorylation was estimated as an end-point assay from comparison of the conversion of the colorimetric substrate to standard curves.

[0018] Molecular Devices "IMAP" technology is reportedly based on the affinity binding of phosphate by immobilized trivalent metal ions on nanoparticles. The IMAP "binding reagent" is said to complex with phosphate groups on phosphopeptides generated in a kinase reaction which would cause a change in the rate of the molecular motion of the peptide, and result in an increase in the fluorescence polarization value observed for a fluorescein label attached at the end of the peptide.

[0019] Pierce's Iron Quench Technology is said to utilize an iron compound that would act as a dark quencher upon specific binding to the phosphoryl group of a fluorescent dye-labeled phosphorylated peptide. The binding event would result in a decrease in the observed fluorescence intensity of the dye-labeled peptide substrate after kinase phosphorylation. The "IQ Reagent" is said to bind specifically to the phosphorylated end product and result in a reduction in observed fluorescence proportional to the extent of phosphorylation of the reaction mixture.

[0020] A multistep labeling method for chemical detection of protein kinases and phosphorylated peptide targets said to be intended for use in chip-based, microfluidic detection systems has been reported. Jeong and Nikiforo have reported a multi-step kinase assay which used a fluorescein labeled Protein Kinase A (PKA) peptide target that had been phosphorylated using an ATP analogue. Nikiforo has recently reported a second method for direct detection of phosphorylation based upon the reaction of thiophosphorylated peptide substrates with biotin-HPDP (N-6[-(Biotinamido)hexyl]-3-(2'-pyridyldithio) propionamide.

[0021] Fluorescence Resonance Energy Transfer (FRET) refers to the radiationless transfer of energy from an excited donor fluorophore to an acceptor fluorophore. Assays using FRET-based detection are said to be based upon the distance separation of the two fluorophores. FRET-peptide assays may utilize peptides that carry a donor fluorophore such as coumarin that is adjacent to an acceptor fluorophore such as fluorescein. Evaluation of both the donor and the acceptor emission wavelengths before and after protease cleavage is said to give an emission ratio which is said to serve as a measure of protease activity. FRET peptides containing phosphorylation sites within overlapping protease cleavage sites have been used to develop a FRET based kinase assay that is reported to be applicable to both tyrosine and serine/threonine kinases and phosphatases.

[0022] Reported cell based assays include the MesaCup assay which is said to be based upon an immunosorbent assay format and to use a synthetic peptide and a monoclonal antibody specific for a phosphorylated form of the peptide substrate. This assay is said to be useful for crude cell extracts and column fractions as well as on purified or recombinant enzymes.

[0023] Published U.S. Patent Application 2002/0068271 to Altman and Coudronniere, reported a cell-based assay said to be specific for the θ isoform of PKC. It is said to be based upon measuring the activity of I β B-kinase β or the activity of an NF κ B. In one aspect the activity of the PKC θ peptide was said to be determined using a phosphorylation assay. In another aspect the activity of the PKC θ peptide was said to be determined using an assay said to be based upon measurement of a reporter construct such as an NF κ B-responsive element, such as a viral promoter, an IL-2 promoter or a CD28RE/AP-1 element. The targets were reported to be isozyme-specific. The methods were said to rely on conventional methods for measurement of phosphorylation.

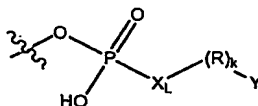
[0024] Some limitations in reported methods for detection of phosphoryl groups on the protein, polynucleotide and lipid target substrates for the kinases and phosphatases may be attributed to the low capability to detect hydroxyl groups. Moreover, the presence of hydroxyl groups that are not targets for kinase or phosphatase activities on cellular components, may present issues regarding specificity in the detection of hydroxyl groups that are uniquely associated with the activities of the enzymes.

[0025] The disclosures of the references cited herein are hereby incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0026] The present methods for detection of phosphorylation and de-phosphorylation of the current invention provide a number of advantages. For example, the present method is not limited by a dependence upon substrate specific IgG molecules for each different peptide substrate. Nor are they troubled by non-specific interactions with other ions in a sample, such as EDTA, vanadate, divalent cations and ATP itself. Furthermore, the present methods are applicable in both homogeneous and heterogeneous assays and are rapid and accurate enough for high throughput screening (HTS) applications.

[0027] One general embodiment of the invention is a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising a group having the formula



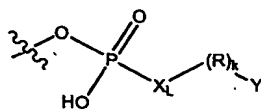
wherein:

X_L is selected from the group consisting of NH, NR_1 and O, wherein R_1 is selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl or heterocycloalkyl group;

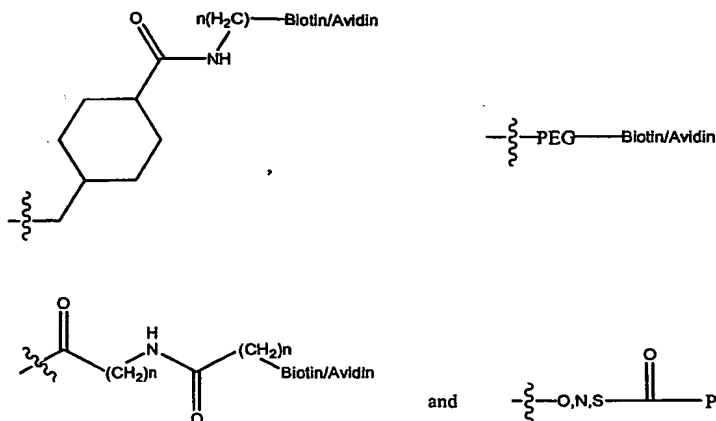
each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-CO-$, $-NHCO-$, $-O-$, $-S-$, $-NR_2CO-$, $-COOR_2-$, $-CONH-$, and $-CONHR_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group. In a further embodiment, each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted heteroalkyl, alkyl, cycloalkyl, heterocycloalkyl, $-CONH-$, $-NHCO-$ and $-CO-$ groups; X_L is NH; k is a whole number from 1-10 and Y is biotin/avidin. In another embodiment, the $-(R)_k-Y$ moiety of the group of the formula

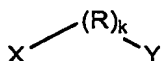


is selected from the group consisting of



Where n is a whole number from 1-25. In yet another embodiment, the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide further comprises a fluorophore.

[0028] Another general embodiment of the invention is a method of covalently attaching one or more hetero-bifunctional linkers having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold,

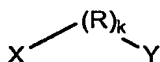
polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group;

to one or more hydroxyl moiety of a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising the steps of:

(a) converting the hydroxyl moiety to phosphoric acid or amino derivative of phosphoric acid; and

(b) converting the phosphoric acid or amino derivative of phosphoric acid to a phosphoro analog by reacting the phosphoric acid or amino derivative of phosphoric acid with the hetero-bifunctional linker. In other embodiments, the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide may comprise a fluorophore. In another embodiment, the hydroxyl moiety can be a 5' hydroxyl group on a oligonucleotide, nucleoside or nucleotide or present on a tyrosine, serine, threonine or histidine group. In yet another embodiment, ATP or [γ -NH₂]-ATP is used in step (a) to convert the hydroxyl moiety to the phosphoric acid or amino derivative of phosphoric acid. In still another embodiment, the phosphoric acid derivative is converted into a more reactive intermediate before it is reacted with the hetero-bifunctional linker to form the phosphoro analog.

[0029] Another general embodiment of the invention is directed to a method of covalently attaching one or more hetero-bifunctional linkers having the formula



as defined above, to a hydroxyl moiety of a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising the steps of:

(a) converting the hydroxyl moiety to a phosphoric acid derivative; and

(b) converting the phosphoric acid derivative to a phosphoro analog by reacting the phosphoric acid derivative with a preformed nucleation reagent comprising a hetero-bifunctional linker. In other embodiments, the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide may comprise a fluorophore. In another embodiment, the hydroxyl moiety can be a 5' hydroxyl group on a oligonucleotide, nucleoside or nucleotide or present on a tyrosine, serine, threonine or histidine group. In yet another embodiment, ATP or [γ -NH₂]-ATP is used in step (a) to convert the hydroxyl moiety to the phosphoric acid or amino derivative of phosphoric acid. In still another embodiment, the phosphoric acid derivative is converted into a more reactive intermediate before it is reacted with the preformed nucleation reagent to form the phosphoro analog. In a further embodiment, the preformed nucleation reagent comprises one or more high molecular weight

reagent conjugated to one or more hetero-bifunctional linker. In a specific embodiment, the high molecular weight reagent is avidin and the hetero-bifunctional linker comprises an amino group and a biotin group.

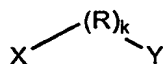
[0030] Yet another general embodiment of the invention is directed to an assay for detecting phosphorylation or de-phosphorylation comprising: (a) a kinase substrate having one or more hydroxyl moiety; (b) a kinase enzyme and ATP or $[\gamma\text{-NH}_2]\text{-ATP}$; and (c) a hetero-bifunctional linker having the formula as defined above. Another general embodiment is directed to an assay for detecting phosphorylation or de-phosphorylation comprising: (a) a phosphatase substrate having one or more hydroxyl moiety; (b) a phosphatase enzyme; and (c) a hetero-bifunctional linker having the formula defined above. Yet another embodiment of the invention is directed to homogeneous assay for detecting phosphorylation or de-phosphorylation comprising: (a) a polypeptide substrate having one or more hydroxyl moiety; (b) a protein kinase enzyme and ATP or $[\gamma\text{-NH}_2]\text{-ATP}$; (c) a monomeric high molecular weight reagent; and (d) a hetero-bifunctional linker having the formula defined above. Still another general embodiment of the invention is directed to a homogeneous assay for detecting phosphorylation or de-phosphorylation comprising: (a) a polypeptide substrate having one or more hydroxyl moiety; (b) a protein phosphatase enzyme; (c) a monomeric high molecular weight reagent; and (d) a hetero-bifunctional linker having the formula defined above.

[0031] Another general embodiment of the invention is a homogeneous assay for detecting phosphorylation or de-phosphorylation comprising: (a) a polypeptide substrate having one or more hydroxyl moiety; (b) a protein kinase enzyme, and ATP an ATP analog or any other phosphate donating group; and (c) a preformed nucleation reagent. In a further embodiment, the preformed nucleation reagent is formed by attaching one or more high molecular weight reagent to one or more hetero-bifunctional linkers having the formula defined above. A further embodiment is directed to a homogeneous assay for detecting phosphorylation or de-phosphorylation comprising: (a) a polypeptide substrate having one or more hydroxyl moiety; (b) a protein phosphatase enzyme; and (c) a preformed nucleation reagent. In a specific embodiment, the preformed nucleation reagent is formed by attaching one or more high molecular weight reagents with one or more hetero-bifunctional linker having the formula defined above.

[0032] Yet another general embodiment of the invention is an assay for detecting phosphorylation or de-phosphorylation comprising the steps of: (a) attaching a fluorophore

to a polypeptide; (b) blocking at least one reactive free amine or acid group on the polypeptide to form a protected polypeptide; (c) phosphorylating the protected polypeptide with ATP, an ATP analog or any other phosphate donating substrate; and (d) measuring fluorescence polarization.

[0033] Still another general embodiment of the invention is a method of preparing a preformed nucleation center comprising the step of reacting one or more high molecular weight reagent with one or more hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group. In another embodiment, the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines and polysulfones. In yet another embodiment, the hetero-bifunctional linker has a reactive amino group. In a further embodiment, the high molecular weight reagent is avidin and the hetero-bifunctional linker comprises biotin.

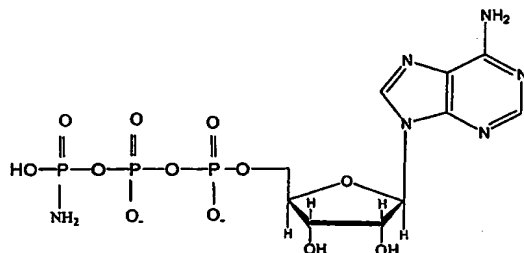
[0034] Another embodiment of the present invention is an assay for the detection of phosphorylation and de-phosphorylation comprising: (a) combining a protein kinase, one or

more test compounds, a labeled polypeptide substrate, and ATP or $[\gamma\text{-NH}_2]\text{-ATP}$ an ATP to make a first mixture; (b) allowing the first mixture to react to form a phosphopeptide product; (c) adding a hetero-bifunctional linker having the formula defined above to create a phosphorylated polypeptide product; and (d) measuring the fluorescence polarization of the phosphorylated polypeptide product. In a further embodiment, the Y group of the hetero-bifunctional linker is biotin. In another embodiment, avidin is added to the phosphorylated polypeptide product before step (d). In yet another embodiment, the mixture comprise a fluorophore.

[0035] In another general embodiment the invention is directed to an assay for the detection of phosphorylation and de-phosphorylation comprising: (a) combining a protein kinase, one or more test compounds, a labeled polypeptide substrate, and ATP or an ATP analog to make a first mixture; (b) allowing the first mixture to react to form a phosphopeptide product; (c) adding a preformed nucleation reagent to create a phosphorylated polypeptide product; and (d) measuring the fluorescence polarization of the phosphorylated polypeptide product.

[0036] In another general embodiment, a preformed nucleation reagent comprising: (a) a high molecular weight reagent; associated with (b) a hetero-bifunctional linker having the formula described above is described.

[0037] Yet another embodiment of the invention is a compound of the formula



BRIEF DESCRIPTION OF THE DRAWINGS

[0038] Figure 1 depicts general compositions and structures of hetero-bifunctional linkers of the present invention. In the Chemistries I, II and III, X can be selected from any number of active groups such as -NH_2 (depicted in chemistry I), succinimidyl (depicted in chemistry II), or the maleimido (BMCC), iodoacetyl, and -SR linkers (depicted in chemistry III).

[0039] Figures 2(a) to 2(d) depict a partial classification of kinases and phosphatases by targets with representative structures:

Figure 2(a) depicts a representative water insoluble target and sites of specific actions of lipid kinases;

Figure 2(b) depicts water soluble target substrates and sites of specific actions of lipid kinases;

Figure 2(c) depicts general peptide target substrate phosphorylation for the actions of the PKC isozymes and their corresponding phosphatases; and

Figure 2(d) depicts typical oligonucleotide target substrate phosphorylation for kinase and phosphatase activities.

[0040] Figures 3(a) and 3(b) are a general diagram and protocol of the single step homogeneous assay method based upon use of a preferred nucleation reagent and the nucleation effect of the present invention. Figure 3(a) depicts a single step homogeneous assay method and Figure 3(b) illustrates the nucleation effect.

[0041] Figures 4(a) and 4(b) depict a general diagram and protocol of the heterogeneous assay method based upon use on a preferred nucleation reagent and the nucleation effect of the present invention. Figure 4(a) depicts a schematic diagram and Figure 4(b) depicts the mechanism of the heterogeneous assay method.

[0042] Figure 5 describes a synthetic scheme for blocking potentially reactive $-NH_2$ and $-COOH$ groups on peptide targets.

[0043] Figure 6 is a Maldi-Mass spectrum demonstrating of the unprotected PKC-peptide target for the kinase activities of the isozymes of PKC.

[0044] Figure 7 describes a synthetic scheme for the formation of phosphoramidates used in the detection of phosphoryl groups using the nucleation centers and rapid assay methods and phosphoramidate Chemistry I.

[0045] Figures 8(a) to 8(c) depict Maldi-Mass spectra of phosphoroamidation of a PKC peptide target by PKC-alpha at the following stages:

Figure 8(a) depicts fully protected fluoresceinated PKC peptide target with potential reactive sites blocked;

Figure 8(b) depicts phosphorylated, fluoresceinated PKC peptide target with potential reactive sites blocked before the addition of multiplexed nucleation centers which were pre-formed from avidin and the hetero-bifunctional biotin linkers of chemistry I; and

Figure 8(c) phosphoramidated, fluoresceinated PKC peptide target with potential reactive sites blocked before the addition of multiplexed nucleation centers that had been preformed from avidin and the hetero-bifunctional biotin linkers of chemistry I.

[0046] Figure 9 depicts an example of fluorescence polarization analysis of the stoichiometry of nucleation center binding. The phosphoramidated PKC peptide target (Figure 7) after the addition of varying amounts of multiplexed nucleation centers using the linkers of Chemistry I.

[0047] Figure 10 shows the chemical structure of the ATP structural analog, γ -Amino ATP (γ -NH₂-ATP).

[0048] Figure 11-1 to 11-3 depict a synthesis of γ -NH₂-ATP.

[0049] Figure 12 depicts a synthetic procedure for phosphoroamidation of fluoresceinated PKC peptide target using PKC-alpha and γ -NH₂-ATP.

[0050] Figure 13 depicts a synthetic procedure for the phosphorothiolation and detection of fluoresceinated PKC-peptide target using the single step, nucleation effect rapid assay method and Chemistry III of the present invention.

[0051] Figures 14(a) to 14(c) depict analytical data for the phosphorothiolation of fluoresceinated PKC peptide target by PKC-alpha:

Figure 14(a) depicts Maldi-Mass spectrum of phosphorothiolated, fluoresceinated PKC peptide target;

Figure 14(b) depicts Maldi-Mass spectrum of the same phosphorothiolated, fluoresceinated PKC peptide target 15 minutes after addition of equimolar equivalents multiplexed nucleation centers prefomed from avidin and the hetero-bifunctional linkers, maleimido BMCC-biotin; and

Figure 14(c) depicts Fluorescence polarization analysis of the same sample used to generate the spectrum in Figure 14b, showing the titration with multiplexed nucleation centers that were prefomed from avidin and the hetero-bifunctional linkers, maleimido BMCC-biotin.

[0052] Figures 15(a) to (c) depict Maldi-Mass spectra of StarBright® Green-PKC peptide target before and after phosphorothiolation by PKC-theta using multiplexed nucleation centers of chemistry III for the reaction:

Figure 15(a) depicts StarBright® Green-PKC peptide target before the addition of enzyme and donor;

Figure 15(b) depicts StarBright® Green-PKC peptide target after the addition of the enzyme and donor and incubation one hour at 32°C;

Figure 15(c) depicts same target as in Figure 15b, one hour after addition of multiplexed nucleation centers preformed from Biotin and the maleimido hetero-bifunctional linkers of Chemistry III.

[0053] Figure 16 shows a Fluorescence Polarization Analysis of the extent of the reaction of multiplexed nucleation centers preformed from avidin and multiple hetero-bifunctional linkers bearing biotin at one terminus and maleimido reactive groups at the other. The StarBright® Green-PKC peptide target was phosphorylated by PKC-theta using γ -S-ATP as the donor.

[0054] Figures 17(a) and (b) depict Maldi-Mass spectra of StarBright® Orange-PKC peptide target:

Figure 17(a) depicts the spectrum before phosphorothiolation; and

Figure 17(b) depicts the spectrum after phosphorothiolation.

[0055] Figures 18(a) and 18(b) depict a Fluorescence Polarization analysis of the stoichiometry of nucleation center binding:

Figure 18(a) depicts fluorescence polarization analysis of the inhibition of PKC-theta phosphorothiolation of StarBright® Orange-PKC peptide target by 5 mMolar EDTA. The multi-plexed nucleation centers used here were preformed from avidin mixed with the hetero-bifunctional linkers bearing biotin-LC-BMCC; and

Figure 18(b) depicts fluorescence polarization analysis of the inhibition of PKC-theta phosphorothiolation of StarBright® Orange-PKC peptide target by 5 mMolar EDTA. The multi-plexed nucleation centers used here were preformed from avidin mixed with the hetero-bifunctional linkers bearing iodoacetyl-LC-biotin.

[0056] Figure 19 depicts results from a Fluorescence Polarization analysis of phosphorothiolated StarBright® Green-PKC peptide target after the addition of multi-plexed nucleation centers comprised of alkaline phosphatase bearing multiple maleimido groups reactive with chemistry III.

[0057] Figure 20 depicts results for a homogeneous assay by fluorescence polarization showing inhibition by Stauro-sporine of the phosphorothiolation of StarBright® Green-PKC target by protein kinase C-alpha. The measurement was made after the addition of multi-plexed nucleation centers that had been preformed as described in the text from avidin and hetero-bifunctional linkers bearing biotin at one terminus and maleimido groups at the other.

DETAILED DESCRIPTION OF THE INVENTION

[0058] The present invention discloses novel homogeneous and heterogeneous assay methods, labeling chemistries and kit configurations that enable rapid covalent reactions with phosphoryl, phosphoramino and phosphorothioate groups, as well as the use of such reactions to rapidly and accurately measure the activities of kinase and phosphatase enzymes.

[0059] The chemistries of the invention enable direct detection of phosphoryl, phosphoramino or phosphorothioate reaction products under (i) liquid phase, homogeneous reaction conditions, in which the reaction substrates are present when the detection of final product is made, or (ii) liquid or solid phase heterogeneous reaction conditions, in which the initial reagents are separated from the final reaction product(s) before detection of the latter.

[0060] In each of these assays, the three chemistries of the present invention employ hetero-bifunctional linkers that attach covalently to phosphoryl, phosphoramino or phosphorothio groups, together with synthetic nucleation centers that speed up the covalent reactions. In liquid phase homogeneous assays in microtiter plates, detection may be based upon changes in the fluorescence polarization of a direct fluorescent label attached to a peptide, lipid or oligonucleotide target substrate which bears phosphoryl, phosphoramino or phosphorothio groups. Contrastingly, in solid or liquid phase heterogeneous assays, the detection reagent is enzyme linked and detection may be based upon changes in the fluorescence emission of a fluorogenic enzyme substrate.

DEFINITIONS

[0061] The following definitions of terms are provided for ease in understanding the description.

[0062] As used herein, the terms "comprising," "including" and "such as" are used in their open, non-limiting sense.

[0063] "Biochemical assays of kinase activities" and "biochemical assays of phosphatase activities" are *in vitro* assay methods conducted using neat solutions of one or more specific kinase or phosphatase enzymes. These *in vitro* assay methods are not performed (i) in intact biological cells, (ii) in biological fluids such as cell lysates or serum, or (iii) in the presence of biological materials, other than those that are directly needed as reagents or components of the assay.

[0064] "Cell-based assays of kinase activities" and "cell-based assays of phosphatase activities" are *in vivo* or *in vitro* assay methods that can be used to detect the activity of one

or more kinase or phosphatase enzymes in either intact biological cells or biological fluids such as cell lysates or serum.

[0065] A “microfluidic device” is a device within which biological or chemical reagents are isolated in separate chambers until they are transferred through channels between chambers. It is possible for the reagents stored in the chambers to participate in a desired reaction once transferred. An example of a microfluidic device is a device which includes (i) at least one fluid storage chamber which has at least one cross sectional dimension between 0.1 and 500 microns, and (ii) at least one channel, which has a cross sectional dimension smaller than the dimension of the chamber, in which reactions and detection can be performed separately from the material in the storage chambers. In one example of a microfluidic device, at least one channel and/or chamber is disposed within the body of the device. The chambers may either be separate and discrete or they may be fluidly connected through the channels, channel intersections and/or valves. Channel intersections may exist in a number of formats, including cross intersections, “T” intersections or any number of other configurations through which two or more channels and/or receptacles are in fluid communication. In biological assay applications, an assay is performed by transferring reagents through channels between wells and only one experiment or assay is performed in each microfluidic device.

[0066] A “microwell” or “microtiter” plate is a device in which biological or chemical reagents are isolated in separate depressions called “wells”. One or more reagents are either used within the same well in performing a desired reaction or transferred by some external means to another unconnected well in the plate where the dried reaction and detection are performed. In biological assay applications, all or part of an assay is performed within a well, and one purpose of the matrix of wells is to enable performance of many such assays at the same time in the same plate.

[0067] “Multi-plexing” is the ability of an assay or measurement to detect and quantify several targets at the same time and “multi-plexed” means any of a range of oligomers, synthetic polymers or biopolymers such as casein, IgG, avidin, glycoproteins or polyethylene glycol, to which one or more hetero-bifunctional linkers have been attached or conjugated to create a complex molecule with a large molecular mass that carries one or more active linkers.

[0068] “Homogeneous nucleation” and “heterogeneous nucleation” in liquids is the random formation of clusters of particles which accelerate chemical and physical processes. The catalyst in an induced nucleation lowers the thermodynamically determined kinetic barrier of the process thereby increasing the rate of nucleation and the kinetics of overall

phase transformation. The formation of new phases within complex liquid mixtures begins with nucleation.

[0069] A "high molecular weight reagent" is any substance which contributes molecular mass. Suitable examples of high molecular weight reagents include avidin, glycosylated proteins such as antibodies, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines, polysulfones, oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, or any large molecule containing at least one group that has been pre-conjugated to one or more appropriate linker (see, e.g., Figure 1) and contains one or more functional group such as maleimides, NHS, esters or hydrazide, capable of covalently reacting with the second linker. In various embodiments, these nucleation and detection reagents may contribute molecular mass, such as in the case of fluorescence polarization measurements, or they may themselves be conjugated to fluorescent labels or enzymes for fluorescent or fluorogenic detection. In specific embodiments, a high molecular weight reagent has a mass of greater than 5,000 daltons.

[0070] "Hetero-bifunctional linkers" are molecules which include at least two different reactive groups in their compositions that covalently bind or conjugate to complementary groups. The different reactive groups on the small molecules do not measurably cross-react with one another. In various embodiments of the invention, one reactive group (first reactive group) binds covalently and specifically with a hydroxyl, amino or sulfhydryl moiety of the phosphoric acid derivative of a kinase or phosphatase target, and another reactive group (second reactive group) undergoes conjugations with other nucleophiles such as amines, sulfhydryls or other bioconjugation groups such as avidin.

[0071] A "nucleation reagent" is composed of at least one high molecular weight reagent, which does not react with the first reactive group on the hetero-bifunctional linker but associates with a second reactive group of one or more hetero-bifunctional linkers to form high molecular weight multi-valent complexes called "nucleation centers." These nucleation centers provide activation centers for process enhancement. In various embodiments of the invention, the formation of new phases within the complex liquid mixtures begins with nucleation, where formed clusters of particles accelerate chemical and physical processes.

[0072] A "preformed nucleation reagent" is formed when a second reactive group of the hetero-bifunctional linker associates with other nucleophiles or bioconjugation groups before the first reactive group of the hetero-bifunctional linker is allowed to react with the phosphoric acid derivative of the target substrate.

[0073] "Signal transduction" describes the transfer of information from the exterior of a cell membrane through the cytoplasm and into the nucleus where the information evokes some change in gene expression.

[0074] "Signal transduction pathways" may involve combinations of kinase and/or phosphatase activities to convey information from the cell membrane to its nucleus. Examples of such combinations include: (1) receptors that penetrate the plasma or intracellular membranes and have intrinsic enzymatic activities such as tyrosine protein kinase and/or phosphatase activities, serine/threonine protein kinase or phosphatase activities and several activators of phosphatidyl inositol. Receptors with intrinsic tyrosine kinase activity are capable of autophosphorylation as well as phosphorylation of other substrates. Additionally, several families of receptors which lack intrinsic enzyme activity can be coupled to intracellular tyrosine kinases by direct protein-protein interactions; (2) receptors that are coupled, inside the cell, to GTP-binding and hydrolyzing proteins ("G-protein coupled receptors" or "GPCR"s). Receptors of the class that interact with G-proteins have structures characterized by seven transmembrane spanning domains. These receptors are termed serpentine receptors. Examples of this class are the adrenergic receptors, odorant receptors and hormone receptors such as angiotensin, vasopressin and bradykinin; and (3) receptors that are found intracellularly and upon ligand binding migrate to the nucleus where the ligand-receptor complex directly affects gene transcription. In general, the polynucleotide kinases and phosphatases are of this type of receptor.

[0075] The term "polypeptide" includes the terms "protein," "polypeptide," and "peptide" as used in their conventional meaning, i.e., a chain of amino acids. None of the terms set length or range of lengths of amino acid chains. These terms also do not imply or exclude post-translation (or post-synthetic) modifications of the polypeptide, e.g., glycosylations, acetylations, phosphorylations, and the like as well as other modifications known to those of skill in the art, both naturally occurring and non-naturally occurring. A polypeptide may be an entire protein or a region of the amino acid chain. A protein may comprise more than one polypeptide.

[0076] The polypeptides, amino acids, nucleosides, nucleotides, oligonucleotides or lipids that are phosphorylated or de-phosphorylated are referred to as "target substrates," "targets" or specifically as "polypeptide substrates," "oligonucleotide substrates" or "lipid substrates." For both kinase and phosphatase enzymes, the target substrate is the compound that is phosphorylated or de-phosphorylated by the action of the enzyme. Additionally, for the kinase enzymes, there is also a "donor substrate" which can be ATP, an ATP analog, GTP or

any other phosphate donor group from which the phosphoryl, phosphoroamino or phosphorothioate group is transferred to the target substrate.

[0077] Examples of suitable "fluorescence detection methods" include (i) molecular intensity, which is the molecular brightness or intensity of the fluorescence emission from a sample over a defined period of time and is typically measured by either signal averaging methods or photon counting methods; (ii) fluorescence polarization, which is the emission of light in a defined plane relative to the plane of the polarized light used to excite fluorophores in a sample; and (iii) fluorogenesis, which is the chemical or physical conversion of a molecule from a state in which it is not fluorescent at a prescribed emission wavelength function into a different state in which is fluorescent at the prescribed wavelength.

[0078] A "fluorophore" refers to a substance, or portion thereof, which is capable of emitting fluorescence in a detectable range. For the fluorescent structural analogs of the nucleotides, this fluorescence typically occurs at wavelengths in the near ultraviolet through the visible wavelengths. In one embodiment, fluorescence occurs at wavelengths between 300 nm and 700 nm. In an alternate embodiment, fluorescence occurs at wavelengths between 300 nm and 500 nm. Suitable examples of fluorophores are those listed in the Molecular Probes Catalogue which is incorporated by reference herein and includes fluorophores such as Alexa Fluor[R] 350, Dansyl Chloride (DNS-Cl), 5-(iodoacetamida)fluorescein (5-IAF); fluorescein 5-isothiocyanate (FITC), tetramethylrhodamine 5- (and 6-)isothiocyanate (TRITC), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), 7-nitrobenzo-2-oxa-1,3,-diazol-4-yl chloride (NBD-Cl), ethidium bromide, Lucifer Yellow, 5-carboxyrhodamine 6G hydrochloride, Lissamine rhodamine B sulfonyl chloride, Texas Red[R] sulfonyl chloride, BODIPY[R], naphthalamine sulfonic acids such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and 6-(p-toluidinyl)naphthalene-2-sulfonic acid (TNS), Anthroyl fatty acid, DPH, Parinaric acid, TMA-DPH, Fluorenyl fatty acid, Fluorescein- phosphatidylethanolamine, Texas red-phosphatidylethanolamine, Pyrenyl- phophatidylcholine, Fluorenyl-phosphotidylcholine, Merocyanine 540, 1-(3- sulfonatopropyl)-4-[[bgr]-[2[(di-n-butylamino)-6naphthyl]vinyl]pyridinium betaine (Naphtyl Styryl), 3,3'dipropylthiadicarbocyanine (diS-C(3)-(5)), 4-(p-dipentyl aminostyryl)-1-methylpyridinium (di-5-ASP), Cy-3 Iodo Acetamide, Cy-5-N-Hydroxysuccinimide, Cy-7-Isothiocyanate, rhodamine 800, IR-125, Thiazole Orange, Azure B, Nile Blue, Al Phthalocyanine, Oxaxine 1, 4', 6-diamidino-2-phenylindole (DAPI), Hoechst 33342, TOTO, Acridine Orange, Ethidium Homodimer, N(ethoxycarbonylmethyl)-6-methoxyquinolinium (MQAE), Fura-2, Calcium Green, Carboxy SNARF-6, BAPTA,

coumarin, phytofluors, Coronene, the StarBright® family of dyes (Chromagen, San Diego, CA), Fluorescein and metal-ligand complexes.

[0079] The term "label" refers to a moiety that, when attached to a target substrate, renders the target substrate detectable using known detection means. Suitable examples of labels include fluorophores, chromophores, radioisotopes, spin-labels, enzyme labels, chemiluminescent labels, and the like, which allow direct detection of a labeled compound by a suitable detector, or, a ligand, such as an antigen, or biotin, which can bind specifically with high affinity to a detectable anti-ligand, such as a labeled antibody or avidin. In one embodiment, the labels are fluorescent dyes such as fluorescein-type or rhodamine-type dyes.

[0080] A "polynucleotide," "oligonucleotide" or "oligomer" is a nucleotide chain structure containing at least two commonly occurring nucleotides or fluorescent structural analogs.

[0081] The term "alkyl" refers to a straight- or branched-chain alkyl group having from 1 to 12 carbon atoms in the chain. Exemplary alkyl groups include methyl (Me, which also may be structurally depicted by /), ethyl (Et), n-propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl (tBu), pentyl, isopentyl, tert-pentyl, hexyl, isohexyl, and the like.

[0082] The term "heteroalkyl" refers to a straight- or branched-chain alkyl group having from 2 to 12 atoms in the chain, one or more of which is a heteroatom selected from S, O, and N. Exemplary heteroalkyls include alkyl ethers, secondary and tertiary alkyl amines, alkyl sulfides, and the like.

[0083] The term "alkenyl" refers to a straight- or branched-chain alkenyl group having from 2 to 12 carbon atoms in the chain. Illustrative alkenyl groups include prop-2-enyl, but-2-enyl, but-3-enyl, 2-methylprop-2-enyl, hex-2-enyl, and the like.

[0084] The term "alkynyl" refers to a straight- or branched-chain alkynyl group having from 2 to 12 carbon atoms in the chain. Illustrative alkynyl groups include prop-2-ynyl, but-2-ynyl, but-3-ynyl, 2-methylbut-2-ynyl, hex-2-ynyl, and the like.

[0085] The term "aryl" (Ar) refers to a monocyclic, or fused or spiro polycyclic, aromatic carbocycle (ring structure having ring atoms that are all carbon) having from 3 to 12 ring atoms per ring.

[0086] The term "heteroaryl" (heteroAr) refers to a monocyclic, or fused or spiro polycyclic, aromatic heterocycle (ring structure having ring atoms selected from carbon atoms as well as nitrogen, oxygen, and sulfur heteroatoms) having from 3 to 12 ring atoms per ring.

[0087] The term "cycloalkyl" refers to a saturated or partially saturated, monocyclic or fused or spiro polycyclic, carbocycle having from 3 to 12 ring atoms per ring.

[0088] A "heterocycloalkyl" refers to a monocyclic, or fused or spiro polycyclic, ring structure that is saturated or partially saturated and has from 3 to 12 ring atoms per ring selected from C atoms and N, O, and S heteroatoms.

[0089] The term "halogen" represents chlorine, fluorine, bromine or iodine. The term "halo" represents chloro, fluoro, bromo or iodo.

[0090] The term "substituted" means that the specified group or moiety bears one or more substituents. The term "unsubstituted" means that the specified group bears no substituents. The term "optionally substituted" means that the specified group is unsubstituted or substituted by one or more substituents. Substituents may be independently selected from the group consisting of alkyl, heteroalkyl, haloalkyl, haloaryl, halocycloalkyl, haloheterocycloalkyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, -NO₂, -NH₂, -N-OH, -N-OR_c, -CN, -(CH₂)_z-CN where z is 0-4, halogen, -OH, -O-R_a-O-, -OR_b, -CO-R_c, -O-CO-R_c, -CO-OR_c, -O-CO-OR_c, -O-CO-O-CO-R_c, -O-OR_c, keto (=O), thioketo (=S), -SO₂-R_c, -SO-R_c, -NR_dR_e, -CO-NR_dR_e, -O-CO-NR_dR_e, -NR_c-CO-NR_dR_e, -NR_c-CO-R_e, NR_c-CO₂-OR_e, -CO-NR_c-CO-R_d, -O-SO₂-R_c, -O-SO-R_c, -O-S-R_c, -S-CO-R_c, -SO-CO-OR_c, -SO₂-CO-OR_c, -O-SO₃, -NR_c-SR_d, -NR_c-SO-R_d, NR_c-SO₂-R_d, -CO-SR_c, -CO-SO-R_c, -CO-SO₂-R_c, CS-R_c, -CSO-R_c, -CSO₂-R_c, -NR_c-CS-R_d, -O-CS-R_c, -O-CSO-R_c, -O-CSO₂-R_c, -SO₂-NR_dR_e, -SO-NR_dR_e, -S-NR_dR_e, -NR_d-CSO₂-R_d, -NR_c-CSO-R_d, -NR_c-CS-R_d, -SH, -S-R_b, and -PO₂-OR_c, where R_a is selected from the group consisting of alkyl, heteroalkyl, alkenyl, and alkynyl, R_b is selected from the group consisting of alkyl, heteroalkyl, haloalkyl, alkenyl, alkynyl, halogen, -CO-R_c, -CO-OR_c, -O-CO-O-R_c, -O-CO-R_c, -NR_c-CO-R_d, -CO-NR_dR_e, OH, aryl, heteroaryl, heterocycloalkyl, and cycloalkyl, and R_c, R_d and R_e are each independently selected from the group consisting of hydrogen, halogen, alkyl, heteroalkyl, haloalkyl, alkenyl, alkynyl, -COR_f, -COOR_f, -O-CO-O-R_f, -O-CO-R_f, -OH, aryl, heteroaryl, cycloalkyl, and heterocycloalkyl, or R_d and R_e cyclize to form a heteroaryl or heterocycloalkyl group, and R_f is selected from the group consisting of hydrogen, alkyl, and heteroalkyl, and where any of the alkyl, heteroalkyl, alkylene, aryl, cycloalkyl, heterocycloalkyl, or heteroaryl moieties present in the above substituents may be further substituted with one or more substituents independently selected from the group consisting of -NO₂, -NH₂, -CN, -(CH₂)_z-CN where z is 0-4, halogen, haloalkyl, haloaryl, -OH, keto (=O), -N-OH, -NH₂, -CO-NH₂, -CO-OH, -NH-CO-NH₂, -C-CO-OH, and unsubstituted alkyl, unsubstituted aryl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, and unsubstituted heteroaryl.

EXEMPLARY CLASSES OF TARGET SUBSTRATES

[0091] Three classes of complex molecular molecules function as target substrates in biological systems for kinase and phosphatase enzymes: polypeptides, oligonucleotides and lipids. For each class, there is a distinct set of enzymes that are class specific, for example, lipid kinases catalyze phosphorylation only of lipid targets. By contrast, within each group of class-specific kinases, there are various levels of target specificity, ranging from sequence specific to promiscuous, as is the case for many oligonucleotide kinases. As a group, all kinase enzymes catalyze the transfer of a phosphoryl group or structural analog of a phosphoryl group from ATP, an ATP analog, GTP or any other phosphate donor to the appropriate target. Phosphatases remove such phosphoryl groups.

[0092] Given the large number of kinases and targets, the protein kinases are generally used here to represent many of the applications of the present invention to all classes of kinase and phosphatase enzymes.

Kinase Enzymes

[0093] The applications of the assay chemistries of the present invention cover all classes of kinase and phosphatase enzymes. Although these quite different enzymes do share some related structural features, for purposes of the assay configurations in which the present invention can be applied, they are generally classified according to the classes of biological molecules upon which they act. Each class may be further subdivided according to their structural domains for catalysis and substrate binding as well as their specificities for prescribed substrate compositions.

Polynucleotide Kinase and Phosphatase Enzymes

[0094] The kinase and phosphatase enzymes associated with DNA phosphorylation are not oligonucleotide sequence specific or even activity specific. In general, the polynucleotide kinases catalyze the transfer of the terminal phosphate of ATP ([[gamma]]-orthophosphate) or the phosphoryl moieties from an ATP analog, to the 5'-hydroxyl termini of deoxyribonucleotides although this activity is quite promiscuous and can also involve RNA, oligonucleotides or nucleoside 3'-monophosphates. The wild type enzymes can also display 3'-phosphatase activities, catalyzing the hydrolysis of 3'-phosphoryl groups of deoxynucleoside 3'-monophosphates, deoxynucleoside 3', 5'-diphosphates and of 3'-phosphoryl-polynucleotides. The basic mechanism of action in the presence of ADP is an

exchange reaction in which an excess ADP causes the enzyme to transfer the 5' terminal phosphate from phosphorylated DNA to ADP. The DNA thus de-phosphorylated may then be re-phosphorylated by transfer of γ -phosphate from [[[gamma]]P]ATP or transfer of the γ -phosphoryl moiety from an ATP analog.

Protein Kinases

[0095] Protein kinases represent the largest superfamily of homologous proteins with over 300 mammalian members known to date and a great number more predicted from genome sequencing and analysis. Despite their involvement in numerous and diverse cellular pathways, the majority of protein kinases share several common sequence and structural motifs. Tertiary structure reveals that the highly conserved region of 250–300 amino acids delineated by sequence similarity, folds into a common catalytic core. The bi-lobal structure formed consists of thirteen conserved subdomains allowing kinases to perform their three main roles, namely, binding and orientation of ATP (or structurally related analogs, including GTP) binding and orientation of the target substrate and phosphate transfer. The smaller N-terminal lobe consists predominantly of anti-parallel beta sheets and is involved in the anchoring of the nucleotide. The larger C-terminal lobe is alpha helical, binds the substrate and initiates transfer of phosphate. The cleft between the lobes is the site of catalysis.

Protein Kinase Subclasses

[0096] In mammalian kinases, two main subdivisions exist based on substrate specificity; Protein Tyrosine Kinases (PTKs) and Serine/Threonine Protein Kinases (S/TKs) with short amino acid stretches characterizing each class. Each class can be further divided into subclasses and subfamilies. Tyrosine Kinases consist of both receptor tyrosine kinases (RTKs) and cytosolic kinases with several families within each group. Three main groups occur in the Serine/Threonine Kinase family. The AGC group includes the cyclic nucleotide-regulated protein kinase families (PKA and PKG) and the diacylglycerol activated / phospholipid dependent (PKC) family and related kinases. The CaMK group of kinases contains the family of kinases regulated by Ca^{2+} /Calmodulin and related subfamilies. The CMGC group contains numerous families including the cyclin dependent kinases (CDK), the MAPK/Erk family as well as the glycogen synthase (GSK-3) and the Clk families.

Receptor Tyrosine Kinases (RTKs)

[0097] The proteins encoding RTKs contain four major structural domains: an extracellular ligand binding domain; an intracellular tyrosine kinase domain; an intracellular regulatory domain; and a transmembrane domain.

[0098] The amino acid sequences of the tyrosine kinase domains of RTKs are highly conserved with those of cAMP-dependent protein kinase (PKA) within the ATP binding and substrate binding regions. RTK proteins are classified into subfamilies based upon structural features in their extracellular portions (as well as the presence or absence of a kinase insert) which include the cysteine rich domains, immunoglobulin-like domains, leucine-rich domains, Kringle domains, cadherin domains, fibronectin type III repeats, discoidin I-like domains, acidic domains, and EGF-like domains. Based upon the presence of these various extracellular domains the RTKs have been sub-divided into at least 14 different families. Many receptors that have intrinsic tyrosine kinase activity as well as the tyrosine kinases that are associated with cell surface receptors contain tyrosines residues, that, upon phosphorylation, interact with other proteins of the signaling cascade. These RTK proteins contain a domain of amino acid sequences that are homologous to a domain first identified in the c-Src proto-oncogene, a non-receptor tyrosine kinase.

Non-Receptor Protein Tyrosine Kinases (PTKs)

[0099] Numerous intracellular PTKs are responsible for phosphorylating a variety of intracellular proteins on tyrosine residues following activation of cellular growth and proliferation signals. Two distinct families of non-receptor PTKs are recognized. The first is the archetypal PTK family, the c-Src gene family with twelve known members believed to be involved in signal transduction, including but not limited to: Hck, Lyn A, Lyn B, Src, Src N1 and Src N2, Fgr, Yrk, Fyn, Lyn, Lck and Blk. The Src protein itself is a tyrosine kinase identified as the transforming protein in Rous sarcoma virus. c-Src ("c"- designates the cellular form of proto-oncogenes that were first identified in transforming retrovirus) is a cellular homolog. The second family is related to the Janus kinase (JAK). Most of the proteins of both families of non-receptor PTKs couple to cellular receptors that lack enzymatic activity themselves. This class of receptors includes all of the cytokine receptors (e.g., the interleukin-2 (IL-2) receptor) as well as the CD4 and CD8 cell surface glycoproteins of T-cells and the T-cell antigen receptor (TCR).

c-Src Tyrosine Protein Kinases

[0100] The c-Src PTKs contain three domains (SH1, SH2 and SH3) which are found in many other signaling proteins. The SH1 domain has PTK activity and the SH2 and SH3 domains are involved in mediating protein-protein interactions by binding to phosphotyrosine-containing and proline-rich motifs, respectively. The interactions of SH2 domain containing proteins with RTKs or receptor associated tyrosine kinases leads to tyrosine phosphorylation of the SH2 containing proteins. The result of the phosphorylation of SH2 containing proteins with enzymatic activity is an alteration (either positively or negatively) in that activity. SH2-containing proteins that have intrinsic enzymatic activity include enzymes in each of the three different classes such as: phospholipase C-g (PLC-g), the proto-oncogene c-Ras associated GTPase activating protein (rasGAP), phosphatidylinositol-3-kinase (PI-3K), protein phosphatase-1C (PTP1C), as well as members of the Src family of protein tyrosine kinases (PTKs).

Receptor Serine / Threonine Kinases

[0101] The receptors for the activins and TGFs- β have intrinsic serine/threonine kinase activity. Although both of these families of proteins can induce and/or inhibit cellular proliferation or differentiation, the signaling pathways utilized are different than those for receptors with intrinsic tyrosine kinase activity or that associate with intracellular tyrosine kinases. One nuclear protein involved in the responses of cells to TGF- β is the proto-oncogene, which directly affects the expression of genes harboring Myc-binding elements.

Non-receptor Serine/Threonine Kinases

[0102] There are several serine/threonine kinases that function in signal transduction pathways, two of which are the cAMP-dependent protein kinases (PKAs) and the protein kinase C isoforms (PKCs). PKC was originally identified as a serine/threonine kinase that was maximally active in the presence of diacylglycerols (DAG) and calcium ion. There are at least ten proteins of the PKC family.

[0103] Each of these enzymes exhibits specific patterns of tissue expression and activation by lipid and calcium. PKCs are involved in the signal transduction pathways initiated by certain hormones, growth factors and neurotransmitters. The phosphorylation of various proteins, by PKC, can lead to either increased or decreased activity. One example is the phosphorylation of the EGF receptor by PKC which down-regulates the tyrosine kinase

activity of the receptor. This effectively limits the length of the cellular responses initiated through the EGF receptor.

[0104] Examples of additional serine/threonine kinases important for signal transduction include the mitogen activated protein kinases (MAP).

Phosphatidylinositol Kinases

[0105] Three classes of PI3Kinases have been identified based upon their primary structures, regulation and substrate specificity. Class I PI3Ks can produce PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ and are regulated by receptor tyrosine kinases and G-protein receptor coupled receptors; Class II PI3Ks can produce PtdIns(3)P, PtdIns(3,4)P₂ and contain a PX homology and C2 domains at their C-terminal regions. The PX domain of 100 to 120 amino acids show the polyproline motif (PXXP) that is characteristic of SH3 domain binding motifs, giving rise to the possibility that PX domains may be interaction partners with the SH3 domain proteins. Perhaps of greater general importance, the PX domains now appear to function as novel motifs for direct interactions with PtdIns3P for membrane targeting and/or regulatory activities.

[0106] There are two known classes of PIPKs: the 68 kD PIP5K (alpha, beta, and gamma species) that generates PtdIns(4,5)P₂ from PtdIns4P, and the 53 kD PIP4K (alpha, beta, and gamma species) that phosphorylates PtdIns3P and PtdIns5P to create PtdIns(3,4)P₂ and PtdIns(4,5)P₂, respectively. The Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma (PIP5KI-gamma), is a member of the PIPKinase family which is also known as PIP5K1C, and KIAA0589. This enzyme catalyzes phosphorylation of the D-5 position of the inositol ring of PtdIns4P (Figure 2).

Lipid Kinase and Phosphatase Enzymes

[0107] The cellular membranes of lipid kinase and phosphatase enzymes not only serve as the structural manifold within which proteins effect signal transduction, but contain lipid constituents within their lipid bilayers that are also key components in many regulatory events. Phosphoinositides are key regulators of diverse cellular processes such as cell proliferation, cell survival, differentiation, signal transduction, cytoskeletal organization and membrane trafficking. The activities of these lipids is regulated by a network of proteins that control their synthesis, transport and degradation such as lipid kinases and lipid phosphatases.

[0108] Phosphoinositide synthesis is regulated by both phosphatidylinositol (PI) kinases and phosphatidylinositol phosphate kinases (PIPKs) whereas PI degradation is regulated by

lipid phosphatases. PI is a collective term for the Phosphatidylinositols (PtdIns) which contain a 1D-myo-inositol phosphate group linked to diacylglycerol (Figure X). The D-myo-inositol head group contains 5 hydroxy groups at positions 2,3,4,5, and 6, three of which, at positions 3, 4 and 5 are known to be targets of phosphorylation by the lipid kinases. These positions may be reversibly phosphorylated singly, doubly or triply, yielding a mixture of phosphorylated PtdIns derivatives. The cellular level of triply phosphorylated PtdIns (3,4,5)P₃ varies dramatically in response to both external and internal regulation; generally, PtdIns(3,4)P₂, PtdIns(4,5)P₂, and PtdIns (3,4,5)P₃ are enriched in the plasma membrane whereas PtdIns (3)P is enriched in the endosomal compartments.

Protein Phosphatase Enzymes

[0109] The same general patterns may be found among the protein phosphatases. Removal of the incorporated phosphates from target substrates is a necessary event in order to turn off proliferative signals. This suggests that phosphatases may function as anti-oncogenes or growth suppressor genes and that the loss of a functional phosphatase involved in regulating growth promoting signals could lead to neoplasia. There are two broad classes of protein tyrosine phosphatases (PTPs): (1) transmembrane enzymes that contain a phosphatase activity domain in the intracellular portion of the protein, and (2) intracellularly localized enzymes. For each of these classes, cell-based assays will ultimately assess the physiological levels of activity.

[0110] There are at least six sub-classes of the transmembrane PTPs: the first transmembrane PTP characterized was the leukocyte common antigen protein, CD45. CD45 is involved in the regulation of the tyrosine kinase activity of Lck, a protein tyrosine phosphatase in T cells, that is a member of the Src family of kinases.

[0111] Lck is associated with the T cell antigens CD4 and CD8 in generating a split-RTK involved in T cell activation. CD45 likely de-phosphorylates a regulatory tyrosine phosphorylation site in the C-terminus of Lck, thereby increasing the activity of Lck towards its substrate(s). The second class of PTPs are the intracellular proteins (interestingly, the CD45 protein has a homology to the intracellular PTP, PTP1B).

[0112] The C-terminal residues of most if not all intracellular PTPs are very hydrophobic and suggest these sites are membrane attachment domains of these proteins. Two intracellular PTPs (PTP1C and PTP1D) contain SH2 domains which, in turn, allow these PTPs to directly interact with tyrosine phosphorylated RTKs and PTKs, thereby, dephosphorylating tyrosines in these proteins. Following receptor stimulation of signal

transduction events, the SH2 containing PTPs are directed to several of the RTKs and/or PTKs with the net effect being a termination of the signaling events by tyrosine dephosphorylation.

[0113] Other phosphatases that recognize serine and/or threonine phosphorylated proteins also exist in cells. These are referred to as protein serine phosphatases (PSPs). At least 15 distinct PSPs have been identified. The type 2A PSPs exhibit selective substrate specificity towards PKC phosphorylated proteins, in particular serine and threonine phosphorylated receptors. Type 2A PSPs are more effective than other PSPs in dephosphorylating RSKs, proteins that are involved in signaling cascades by phosphorylating ribosomal S6 protein. However, a type 1 PSP is required to dephosphorylate S6 itself. The type 2A PSPs have 2 subunits (a regulatory and a catalytic), both of which can associate with one of the tumor antigens of the DNA tumor virus, polyoma.

[0114] Transformation by DNA tumor viruses such as polyoma appears to be mediated by the formation of a signal transduction unit consisting of a virally encoded T antigen and several host encoded proteins. Several host proteins are tyrosine kinases of the SRC family. Polyoma middle T antigen can also bind to PI-3K. The association of type 2A PSPs in these complexes may lead to de-phosphorylation of regulatory serine/threonine phosphorylated sites resulting in increased signal transduction and subsequent cellular proliferation.

EXAMPLES OF CONJUGATION CHEMISTRY

[0115] In a general embodiment of the invention, covalent phosphoramidate conjugation chemistry is used to detect phosphorylation of target substrates using ATP, an ATP analog or any other phosphate donor as the donor substrate.

Phosphoroamidate Linker Chemistry I (Figure 7)

[0116] In one embodiment of the invention, covalent phosphoroamidate conjugation chemistry for detecting phosphorylation of target substrates uses ATP as the phosphate donor substrate. In this embodiment, ATP converts a hydroxyl group on a target substrate to phosphoric acid. In one specific embodiment, the target substrate is an oligo or lipid class of targets (Figure 2a and 2c). In another specific embodiment, the target substrate is the PKC isozymes wherein adducts which react undesirably with ATP or the fluorescent label, and thus interfere with the action of a kinase or phosphatase or with the subsequent assay, are protected (Figure 2b).

[0117] Figure 5 and Figure 6 provide specific examples where the internal $-NH_2$ groups of lysine and arginine present on the peptide target are protected to prevent these types of undesired reactions.

Phosphoroamino Linker Chemistry II (Figures 8-14)

[0118] In another embodiment of the invention, the phosphoroamino chemistry employs a novel phosphoroamino analog of ATP, $[\gamma-NH_2]$ -ATP, and the corresponding covalent phosphoroamino conjugation chemistry for detecting phosphorylation or de-phosphorylation of target substrates using hetero-bifunctional linkers in combination with purified recombinant or native enzymes.

[0119] The method of this embodiment is based upon aminophosphorylation of targets using $[NH_2]$ -ATP. The synthesis to make $[NH_2]$ -ATP is shown in Figure 8, and an example using $[NH_2]$ -ATP to measure the extent of phosphorylation of a peptide, oligonucleotide or lipid target is generally presented in Figure 9.

Phosphorothioate Linker Chemistry III (Figures 15-18)

[0120] In yet another embodiment of the invention, the phosphorothioate chemistry employs a novel phosphorothioate analog of ATP, $[\gamma-S]$ -ATP, and the corresponding covalent phosphorothioate conjugation chemistry for detecting phosphorylation or de-phosphorylation of target substrates using hetero-bifunctional linkers in combination with purified recombinant or native enzymes.

[0121] In one embodiment, this detection method utilizes a reaction mixture which comprises:

- (i) a kinase or phosphatase substrate containing one or more hydroxyl moiety that can be phosphorylated by kinase enzymes or one or more phosphoryl group that can be de-phosphorylated by phosphatase enzymes, and which may be in a fluid phase or immobilized through covalent attachment to a solid phase such as a microtiter plate or a microfluidic device;
- (ii) a kinase enzyme and $[\gamma-S]$ -ATP, or a phosphatase enzyme; and
- (iii) a hetero-bifunctional linker with a phosphorothioate linker group such as a hetero-bifunctional linker containing an iodoacetate or a maleimide group at one terminus and a different conjugation chemistry group such as biotin at a second terminus.

[0122] In a further embodiment, a detection reagent that reacts specifically with the second conjugation chemistry group is reacted with the linker's second terminus before or during the step in which the phosphorothiate linker is covalently attached to the target substrate through the phosphorothiate group.

Multi-valent Nucleation Centers

[0123] In numerous embodiments of the invention, enhancement of phosphorylation and de-phosphorylation reactions came from the creation of synthetic multi-valent nucleation centers by use of a nucleation reagent. These preformed multi-valent nucleation centers of the three labeling chemistries for phosphoryl groups or their structural analogs, discussed above, has been applied to the development of rapid homogeneous and heterogeneous methods for measuring the phosphorylation, phosphoroamination or phosphorothiolation of targets by all classes of kinase and phosphatase enzymes, including protein, lipid and oligonucleotide. Homogeneous and heterogeneous assay methods capable of utilizing synthetic nucleation centers include, for example, those performed in microwell plate format, array format, microfluidic devices, microtiter format as well as various assay kits.

[0124] Especially in the fluid phase, the reaction of hetero-bifunctional linkers with phosphorylated targets is much slower if reagents are monomeric when added. Thus, the use of preformed nucleation reagents greatly increases these types of reactions.

[0125] In one embodiment of the invention, covalent conjugates of hetero-bifunctional linkers with casein were preformed and used as a single reagent in the one step method method, discussed below. In this reaction, the overall time of the method was reduced from an overnight process to one that was completed in two to three hours.

[0126] In other embodiments of the invention, bioconjugations systems, such as lectin/carbohydrate or biotin/avidin are used as a reagent to form the desired nucleation reagents. In some embodiments, the individual reagents used to form the nucleation reagents are kept separate until the day of use. This eliminates the need to preform the nucleation reagents in a manufacturing process and thereby eliminates quality assurance and quality control concerns when the nucleation reagents will not be used within a short period of time.

[0127] In other embodiments of the invention, the nucleation reagents are fluorogenic. In particular embodiments, after unreacted nucleation reagents are removed, a fluorogenic enzyme substrate is added and fluorescence is measured.

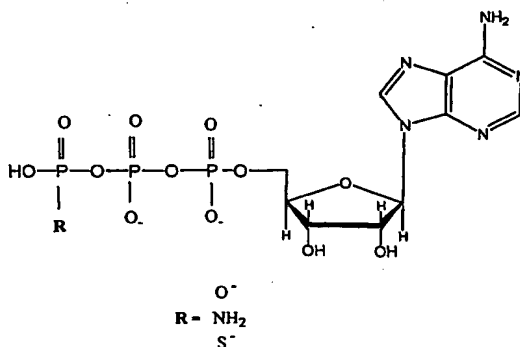
[0128] In a specific embodiment of the invention, the nucleation reagent comprises a hydrophobically bound avidin-biotin-hetero-bifunctional linker complex. In specific

embodiments the hetero-bifunctional linker comprises a conjugation group such as a reactive amine, sulfur, maleimido, iodoacetyl, NHS or ester.

[0129] In another embodiment, the hetero-bifunctional linker is succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-6-amidocaproate or succinimidyl-3-(bromoacetamido)-propionate, which is conjugated to avidin, streptavidin or another enzyme or protein. In further embodiments, the enzyme component is prepared in a 40 fold excess of the linker.

Examples of Phosphoryl Donors

[0130] In one general embodiment, use of a phosphoryl donor such as adenosine triphosphate (ATP), adenosine-5'-O-(3-aminotriphosphate) (γ -NH₂-ATP), and adenosine-5'-O-(3-thiotriphosphate) (γ -S-ATP), of the chemical structures shown below, to develop homogeneous and heterogeneous assays for monitoring the activity of protein kinases and phosphatases is described.



General Structure of Phosphoryl Donors of the Present invention

[0131] The use of ATP and ATP analogs provide for methods that enable rapid, quantitative detection of phosphoro, phosphoroamino or phosphorothioate groups on the protein, oligonucleotide or lipid targets of kinase and phosphatase enzymes.

StarBright® Dye Technology for Fluorescence Detection

[0132] The StarBright® dyes are available from Chromagen and are disclosed in U.S. Provisional Patent Application No. 60/413,025. These dyes can be used as either ultra-sensitive direct fluorescent labels or as fluorogenic enzyme substrates. Fluorogenic substrates are compounds that are converted to fluorescent molecules by enzymes that have been attached to nucleic acid probes or peptides. StarBright® Dye compounds are well suited for detection of polypeptides, lipids, amino acids, nucleosides, nucleotides or

oligonucleotides in biological applications. The chemical composition of the StarBright® family of dyes results in highly polar molecules, which make them water-soluble and reduces in-solution "stacking" of the dye molecules.

[0133] StarBright® dyes include Red, Orange, Green, Blue and Yellow for the spectral wavelengths at which they emit light, which correspond to catalog numbers. Characteristics of the StarBright® dyes include low molecular weight (400-700 daltons); short excited state lifetimes; large quantum yields and extinction coefficients; high solubility and stability in aqueous solutions; large stoke shifts (usually 50nm separation between excitation and emission wavelengths) for efficient multi-target analysis and low background interference; and no photoquenching or photobleaching upon excitation.

[0134] StarBright® substrates enhance fluorescent signals using enzyme-labeled secondary detection reagents in conjunction with fluorogenic substrates. Several of the StarBright® dyes have been derivatized to function as fluorogenic enzyme substrates, which are non-fluorescent until converted to a fluorescent product by enzymatic cleavage. StarBright® substrate are available for enzyme-linked detection systems such as alkaline phosphatase, b-Galactosidase, glucose oxidase and b-lactamase.

ASSAYS FOR DETECTION OF PHOSPHORYLATION AND DE-PHOSPHORYLATION

[0135] Determination of activity of protein kinases presents some of the most difficult analytical challenges of all the kinase and phosphatase enzymes. In higher eukaryotes, thousands of protein kinases have evolved, each with the ability to be activated under specific cellular conditions, and each capable of phosphorylating unique subsets of cellular targets. Studies of the activities of individual kinase and phosphatase enzymes typically have one of two different objectives:

- (i) *in vitro* biochemical assays used in screening chemical libraries to identify drug candidates that specifically promote or inhibit the activities of a specific kinase, or
- (ii) measuring the changes in the activity of a specific kinase in whole cells or their lysates which can be either *in vitro* or *in vivo*, but are cell based and involve measurements in the presence of biological fluids.

[0136] *In vitro* screening or HTS assays are often performed under neat conditions using biochemical assays isolated from recombinant enzymes and known target substrates. By contrast, cell-based assays are usually performed on intact cells, cell lysates or serum samples in which many kinase enzymes and other cellular components may be present and the target substrates may be determined by the spatial location of the particular enzyme

within the cell. Whether biochemical or cell-based assays are used, the need to screen large libraries of chemical compounds to identify chemical leads with therapeutic potential, or screen large numbers of cells to validate population effects, suggests that the ideal assay must be rapid, accurate and compatible with the automated formats currently used in the pharmaceutical industry.

Example of General Assay

[0137] In one embodiment of the invention, a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide is labeled with a fluorophore, such as a StarBright® Dye, Flurescein or Texas Red, to form a labeled polypeptide (Figure 5, compound 2). The labeled polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide is then reacted with a protecting group, such as a thioisocyanate, which attaches to one or more free amines to form an amine protected polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide (Figure 5, compound 3). The terminal acid group of the amine protected polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide is then reacted with a coupling reagent, such as EDC, and an amine which protects the acid of the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide by forming an amide (Figure 5, compound 4). One or more hydroxyl group on the labeled polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide is then reacted with a phosphate donor, such as ATP, [γ -NH₂]-ATP or [γ -S]-ATP, to form a phosphoric acid derivative of the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide (Figure 7, compound 5). Optionally, this phosphoric acid derivative is converted into a more reactive intermediate by reaction with N-Me-Imidazole and a coupling reagent, such as EDC. The phosphoric acid derivative or the reactive intermediate formed from the phosphoric acid derivative is then reacted with a hetero-bifunctional linker (Figure 7, compound 6). Alternatively, the hetero-bifunctional linker may be prereacted with a high molecular weight reagent, such as avidin, to form a nucleation reagent which is subsequently added to the phosphoric acid derivative or the reactive intermediate. Following addition of the hetero-bifunctional linker or the nucleation reagent, fluorescence polarization is measured.

Homogeneous Assay For Detection Of Protein Kinase Activity

[0138] In one general embodiment of the invention, an *in vitro* homogeneous assay embodying the hetero-bifunctional linkers (Figure 1) and phosphoramidate chemistry (Figure

7) is used to detect protein kinase activity. In a specific embodiment, the protein kinase is the PKC isozyme. These *in vitro* homogeneous assay comprise:

(i) a peptide target substrate with hydroxyl moieties that can be phosphorylated by kinase enzymes or de-phosphorylated by phosphatase enzymes within its amino acid sequence (see, e.g., Figure 2b). Specific to protein kinases, the target peptides terminal acid and internal amine moieties can be chemically protected to prevent undesirable reactions that could inhibit the action of the enzyme. In further embodiments, amine and acid protection is accomplished through covalent derivatizations of the protein (see, e.g., Figures 5 and 6);

(ii) a protein kinase enzyme and ATP, an ATP analog or any other phosphate donor, or a protein phosphatase enzyme; and

(iii) either the reagents described below for the Two Step Reaction Method or the reagents described below for the One Step Reaction Method.

Two Step Reaction Method:

(a) Hetero-bifunctional linker with a first reactive group and any number of other reactive groups ("second linker") capable of a different type of conjugation than the first reactive group such as those involving biotin/avidin reactions, lectin/glycoside reactions or chemically reactive groups such as -SH, -COOH or aldehydes, on at least one other terminus (see, e.g., Figures 1 and 7); and

(b) Monomeric high molecular weight reagent which does not react with the reactive groups, but attaches covalently to the hetero-bifunctional linker on the phosphoryl groups and reacts specifically with the second linker groups on the hetero-bifunctional linker after the hetero-bifunctional linker has reacted with the target phosphoryl to form a high molecular weight complex.

One Step Reaction Method:

(a) This one step reaction method protocol is a single step process based upon the formation and use of preformed nucleation centers (see, e.g., Figure 3a). According to one aspect, a preformed high molecular weight nucleation reagent may comprise the reaction product of multiple hetero-bifunctional linkers, each with a first reactive amino group, which are conjugated through their second linker group(s) to form a multi-valent complex with the high molecular weight reagent. This high molecular weight reagent does not react with the reactive groups on the hetero-bifunctional linkers attached covalently to the phosphoryl groups, but rather binds many copies of the linkers to form the high molecular weight multi-valent complexes ("nucleation centers"). These nucleation centers bear multiple hetero-bifunctional linkers on their surfaces.

Homogeneous Assay For Detection Of Lipid Kinase Activity

[0139] In another general embodiment of the invention, an *in vitro* homogeneous assay embodying hetero-bifunctional linkers and phosphoramidate chemistry (Figures 1 and 7) is used to detect Lipid Kinase activity. In a specific embodiment, the lipid kinase is Phosphatidyl Inositol Kinase and the hetero-bifunctional linkers and phosphoroamidate chemistry is used with water soluble StarBright Dye-labeled forms of the phosphatidyl inositor or the corresponding StarBright Dye-labeled myo-inositol. The reaction mixtures of this embodiment comprise:

- (i) a water soluble lipid target substrate (see, e.g., Figure 2b) comprising at least one fluorescently labeled fatty acyl chain covalently attached to a water soluble StarBright® Dye label such as StarBright® Green Dye, and an inositol group comprising a hydroxyl group which can be either phosphorylated by polyinositide kinase enzymes or de-phosphorylated by the corresponding phosphatase enzymes;
- (ii) a lipid kinase enzyme and ATP, an ATP analog or any other phosphate donor, or a lipid phosphatase enzyme; and either
- (iii) reagents for the One Step Reaction Method (described above); or
- (iv) reagents for the Two Step Reaction Method (described above).

Homogeneous Assay For Phosphatase Detection

[0140] In another general embodiment, the invention describes a homogeneous phosphatase detection method comprising a fluid phase which contains:

- (i) a kinase enzyme;
- (ii) a reaction buffer; and
- (iii) a fluorescent labeled peptide, lipid or oligonucleotide target from which the phosphatase enzyme can remove phosphoryl, phosphoroamino or phosphorothioate groups at specific hydroxyl groups on amino acids, lipids or nucleotides. The de-phosphorylating, de-phosphoroaminating or de-phosphorothiolating targets for the phosphatase can be ATP, an ATP analog or any other known phosphate donor capable of acting as the donor reagent for the kinase in phosphorylating the peptide, lipid or oligonucleotide target.

[0141] In a further embodiment, following an incubation period during which the kinase enzyme catalyzes phosphorylation, reactive linkers, such as those which react covalently with phosphoryl, phosphoroamino or phosphorothioate groups, are added in a form in which they are multi-plexed to form a nucleation center. The nucleation center is capable of

substantially increasing both (i) the rate of the covalent reactions of the reactive linkers with the targets, and (ii) the molecular mass of the fluorescent peptide, fluorescent lipid or fluorescent oligonucleotide targets, if the latter have not been de-phosphorylated, de-phosphoroaminated or de-phosphorothiolated by the action of the phosphatase.

[0142] As used herein, the phrase "substantially increased" means that the molecular mass of the final product differs from that of the fluorescent target by an amount sufficient to enable the differentiation of the final product from the initial target through the changes in its fluorescence polarization and to do so rapidly enough to make the detection practical for high throughput screening.

[0143] In a further embodiment, the nucleation center is a polymeric carrier such as casein and the reactive linkers are conjugated to the polymeric carrier by methods known to one skilled in the art.

[0144] In another embodiment, the nucleation center is comprised of avidin which is prereacted with hetero-bifunctional reactive linkers having a covalently attached biotin at one end, and one of the reactive phosphoramidate chemistries I, II or III at the opposite end. By pre-forming the nucleation centers prior to their addition to the reaction mix, it is possible to develop methods for rapid, accurate measurement of phosphoryl groups by substantially accelerating the rates at which phosphorylated targets react covalently with the phosphoryl reactive chemistries I, II or III.

[0145] In yet another embodiment, the nucleation reagent is a polymeric carrier such as a peptide or a glycoprotein and the reactive linkers may be conjugated to the polymeric carrier by any of a number of conjugation chemistries known to one skilled in the art. In a specific embodiment, the nucleation reagent is avidin which is prereacted with hetero-bifunctional reactive linkers having biotin covalently attached at one end and the phosphoryl reactive phosphoramidate chemistries I, II or III at the opposite end. By pre-forming the nucleation reagents prior to their addition to a reaction, molecules which can substantially increase the speed at which targets, that remain phosphorylated, phosphoroaminated or phosphorothiolated, covalently react are formed. This results in an assay which is quantitative, reproducible and practical for use in HTS assays.

[0146] The use of nucleation reagents to speed up covalent reactions between a target substrate and the hetero-bifunctional linkers makes it possible to rapidly and accurately measure the kinetic activity of a kinase or phosphatase through direct covalent reaction of covalent linkers with phosphoryl, phosphoroamino or phosphorothiol groups.

[0147] In other embodiments of both the kinase and the phosphatase homogeneous assays, the fluorescence polarization of the final product is compared to the fluorescence polarization of the first reagent mixture in a single step method. The amount of fluorescence that is emitted from molecular mass changes in a labeled peptide or oligonucleotide during covalent reaction with a high molecular mass nucleation reagent can be estimated directly through comparison with standard curves from solutions containing known amounts of initial reagents and final product. In further embodiments of this invention, the fluorescence polarization of peptide reagent and the final product differ by at least 80 and as much as 300 mP units within two hours of addition of the linker.

Homogeneous Assays in Microwell Plate Format

[0148] In another general embodiment of the invention, the linker chemistries I or II are incorporated into a fluid phase homogeneous microwell-based assay. These assays are useful for profiling the effects of a panel of known enzyme inhibitors or screening the effects of compounds in a chemical libraries on a specific kinase or phosphatase enzyme or isoenzyme, which may be either naturally occurring or recombinant. In one embodiment, the assays enable rapid detection and measurement of phosphoryl groups on fluorescent-labeled target peptides, oligonucleotides or lipids by (i) transfer of a phosphoryl group onto, or off of, the target by a specific kinase or phosphatase, followed by (ii) covalent conjugation of any phosphorylated targets with hetero-bifunctional linker(s) on a nucleation center reagent by use of the phosphoramidate or phosphoramino chemistry. In yet another embodiment of the invention, the number of phosphoryl groups associated with the fluorescent-labeled target are measured after the reaction with the nucleation centers using fluorescence polarization analysis or fluorogenic detection.

Homogeneous Assays in Array Format

[0149] In a further general embodiment of the invention, the linker chemistries I or II are used in fluid phase homogeneous microwell-based assays for profiling the effects of known or potential inhibitors on a panel of purified kinase or phosphatase enzymes. In this embodiment, the extent of phosphorylation of more than one target substrate by either a single kinase or a panel of one or more target substrates, by a panel of kinases, are simultaneously detected in microtiter plate arrays by using the phosphoramidate or phosphoramino linkers in fluid phase homogeneous assays through covalent conjugation of the phosphoramidate or phosphoramino linker.

[0150] Depending upon the number of different fluorescent peptide, lipid or oligonucleotide targets in a well, one to four enzymes can be characterized per sample. At any time before, during or after the conjugation, any of several detection reagents is conjugated with the conjugation chemistry at each linker's second terminus and used with any of several detection methods such as fluorescence polarization analysis, molecular brightness and fluorogenic detection.

Homogeneous Assays in Microfluidic Devices

[0151] In another general embodiment of the invention, hetero-bifunctional linkers for use in the phosphoramidate or phosphoroamino linker chemistry are present in fluid phase homogeneous assays in microfluidic devices. These assays are useful for profiling the effects of a panel of inhibitors or a chemical library on a prescribed kinase enzyme or isoenzyme, which may be naturally occurring or recombinant, by covalent conjugation of the phosphoramidate or phosphoroamino linker.

[0152] At any time after the phosphoramidate or phosphoroamino conjugation, any of several nucleation and detection reagents may be conjugated to the phosphoryl groups through the phosphoramidate or phosphoramino conjugation chemistry and used with a detection method such as fluorescence polarization analysis and fluorogenic detection.

Liquid Phase Homogeneous Phosphorothioate Assays in Microtiter Formats

[0153] In a general embodiment of the invention, hetero-bifunctional phosphorothiolating reagents and preformed nucleation centers are used in fluid phase homogeneous, microwell-based assays. In one embodiment, these assays are used for profiling the effects of a panel of inhibitors or a chemical library on a prescribed kinase or phosphatase enzyme or isoenzyme, which may be naturally occurring or recombinant, by covalent conjugation of the phosphorothioate linkers on nucleation centers to phosphorylated substrates. At any time before, or during the phosphorothioate linker conjugation, any of several high molecular weight reagents are conjugated with the reactive groups at the linker's second terminus to create nucleation centers which are then reacted with phosphorothiolated targets using the rapid reaction method. In another embodiment, the extent of phosphorylation is then determined using a detection method such as fluorescence polarization analysis, molecular brightness, and fluorogenic detection which measures the activity of the enzyme.

Liquid Phase Homogeneous Phosphorothioate Assays Used in Array Formats

[0154] In another general embodiment of the invention, hetero-bifunctional phosphorothiolating reagents and linker chemistry are used in fluid phase homogeneous microwell-based assays. In one embodiment, these assays are used for profiling the effects of known or potential inhibitors on a panel of purified kinase or phosphatase enzymes by simultaneously detecting the extent of phosphorothiolation of substrates in microtiter plate arrays of fluid phase, homogeneous assays. At any time before or during phosphorothioate conjugation, one or more high molecular weight reagents are conjugated with the reactive groups at the linker's second terminus to create nucleation centers. These nucleation centers are then reacted with phosphorothiolated targets using the rapid reaction methods. In a further embodiment, the extent of phosphorylation is determined by a detection method such as fluorescence polarization analysis, molecular brightness and fluorogenic detection which measure the activity of the enzyme.

Liquid Phase Homogeneous Phosphorothioate Assays Used in Microfluidic Devices

[0155] In yet another general embodiment of the invention, hetero-bifunctional phosphorothiolation reagents and linker chemistries are used in fluid phase homogeneous assays in microfluidic devices. In various embodiments, these assays are used for profiling the effects of a panel of inhibitors or a chemical library on a prescribed kinase enzyme or isoenzyme, which may be naturally occurring or recombinant. At any time before or during the phosphorothioate conjugation, one or more high molecular weight reagent is conjugated with the reactive groups at the linker's second terminus to create nucleation centers. In further embodiments, these nucleation centers are then reacted with phosphorothiolated targets using the rapid reaction method and the extent of phosphorylation is determined using a detection method such as fluorescence polarization analysis, molecular brightness or fluorogenic detection to measure the activity of the enzyme.

Heterogeneous Assay Reagents and Methods

[0156] In another general embodiment of the invention, ultrasensitive method of quantitatively measuring the extent of phosphorylation, phosphoramination or phosphorothiolation of peptide, lipid or oligonucleotide targets after the catalytic action of kinase or phosphatase enzymes in cell lysates or other biological fluids are described. In these embodiments, the assay formats are heterogeneous, requiring separation of reagents and reaction products, and utilize the sensitivity of enzyme-linked fluorogenic assays to attain the

sensitivity needed to detect low levels of enzyme activities in cells and cell lysates. In these methods, two types of nucleation centers are employed both of which employ the enzyme that is used in fluorogenesis as the core reagent for forming one of two types of Nucleation Center: (i) Type A Nucleation Centers in which an enzyme is multi-plexed to one or more linkers bearing at their opposite ends one of the reactive chemistries I, II, or III of the present invention, or (ii) Type B Nucleation Centers in which an enzyme is multi-plexed to one or more bioconjugation chemistries that react with a complementary moiety on a hetero-bifunctional linkers.

Heterogeneous Assays For Kinase Detection

[0157] In a further general embodiment, the heterogeneous kinase detection method comprises first providing a solid phase which contains: (i) a reaction buffer, (ii) ATP or an ATP structural analog (such as those of Chemistries II and III) that are known to act as a phosphorylating, phosphoraminate or phosphorothiolating reagent for kinase activities, and (iii) a non-fluorescent and non-phosphorylated, non-phosphoraminate or non-phosphorothiolated peptide, lipid or oligonucleotide target that is covalently or otherwise conjugated to a solid phase such as the thermoplastic of a microtiter plate or a bead. The phosphorylating, phosphoraminate or phosphorothiolating reagent for the kinase is ATP or a structural analog of ATP which is used as the donor reagent for the action of the kinase in phosphorylating, phosphoraminate or phosphorothiolating the peptide, lipid or oligonucleotide target that is bound to the solid phase. In all cases the target and solid phase must be capable of being washed during an assay, without significant loss of the target. This removes unreacted reagents or reaction products that might otherwise interfere with subsequent steps in the assay or the detection.

[0158] In a specific embodiment, PKC isoenzymes are used and kinase activities measured were added as cell lysates, in serum or in other biological fluids, to wells of a microtiter plate to which one or more peptide targets has been previously been conjugated. In this specific embodiment, the peptide target may be one known to be specific for a PKC isoform or may be a general substrate for many different PKC isoforms.

[0159] In various embodiments of the invention which employ Type A Nucleation Centers, following an appropriate incubation period during which one or more kinase enzymes, if present, can catalyze phosphorylation, phosphoramination or phosphorothiolation of the target, the appropriate hetero-bifunctional linkers, capable of covalently reacting with phosphoryl, phosphoroamino or phosphorothioate groups on the target are mixed with the

peptide, lipid or oligonucleotide targets and react with the latter if they have been phosphorylated, phosphoroaminated or phosphorothiolated by the kinase. At their opposite ends, each of the linkers is a component of a preformed Type A Nucleation Centers. In one embodiment, the enzymes used in fluorogenic detection are also used to create the Nucleation Centers. By using such preformed Type A Nucleation Centers, the rate at which the enzyme is bound to phosphorylated, phosphoramined or phosphorothiolated targets is substantially increased. The enzymes used in creating the Type A Nucleation Centers include, but are not limited to, alkaline phosphatase, β -galactosidase, β -glucuronidase, β -lactamase or horse-radish peroxidase.

[0160] In yet a further embodiment using the Type A Nucleation Centers, after a defined reaction period, the plate is washed to remove unreacted nucleation centers and a fluorogenic enzyme substrate specific for the enzyme is added. After an appropriate time interval the activity of the kinase is measured.

[0161] In various embodiments of the invention which employ Type B Nucleation Centers, following an appropriate incubation period during which one or more kinase enzymes, can catalyze phosphorylation, phosphoramination or phosphorothiolation of the target, the appropriate hetero-bifunctional linkers, capable of reacting covalently with phosphoryl, phosphoroamino or phosphorothioate groups on the target, are mixed with the peptide, lipid or oligonucleotide targets that are bound to the solid phase to react with the latter if they have been phosphorylated, phosphoroaminated or phosphorothiolated by the kinase. At their opposite ends, each of the linkers bears a bioconjugating reagent. This bioconjugating reagent is unreactive with the phosphoramidate, phosphoraminate or phosphorothioate chemistries such as biotin, chelating divalent electrostatic linkers such as Nickel, Histidine, phenylboronic acid, salicylhydroxamic acid, or any of a number of lectins. In one embodiment, the Type B Nucleation Centers are pre-formed by covalent combination of an enzyme with one or more such bioconjugation reagents. By such multiplex formation, the rate at which the enzyme is bound to covalently attached hetero-bifunctional linkers is substantially increased. In further embodiments, after a defined reaction period involving the linkers and target substrate, the plate is washed, and the corresponding Type B Nucleation Centers added and allowed to react with the free end of the hetero-bifunctional linker. In a variation of this embodiment, the Type B Nucleation Centers can be pre-formed and reacted with the hetero-bifunctional linkers to create nucleation centers that are a hybrid of the Type A and Type B Nucleation Centers. In either embodiment, once the reaction(s) between the

hetero-bifunctional linkers, targets and the nucleation centers is complete, the solid-phase is washed to remove unreacted reagents. In a further embodiment, a specific fluorogenic substrate for the enzyme is then added to the sample, and after an appropriate time enzyme activity is detected. In a specific configuration of the Type B Nucleation Center, the hetero-bifunctional linker is biotinylated at one end and the corresponding complement is an avidin-enzyme conjugate such as avidin-alkaline phosphatase, avidin β -galactosidase, or avidin- β -galactosidase, avidin- β -glucuronidase, avidin- β -lactamase or avidin-horse-radish peroxidase.

Heterogeneous Assay For Phosphatase Detection

[0162] In a further general embodiment of the invention, the heterogeneous phosphatase detection method comprises a solid phase assay which contains (i) a reaction buffer, and (ii) a fully phosphorylated, fully phosphoraminate or fully phosphorothiolated peptide, lipid or oligonucleotide that is covalently or otherwise conjugated to a solid phase such as the thermoplastic of a microtiter plate or a bead. The fully phosphorylated, phosphoraminate, or phosphorothiolated peptide, lipid or oligonucleotide functions as a target and donor for a phosphatase enzyme. This enzyme can catalyze removal of phosphoryl, phosphoramino or phosphorothioate groups at specific amino acids and lipid components such as inositol sugars or nucleotides. In various embodiments of these assays, the phosphorylated, phosphoraminate or phosphorothiolated peptides, lipids or oligonucleotides are the donor reagents for the action of the phosphatase which catalyzes de-phosphorylation, de-phosphoramination or de-phosphorothiolation.

[0163] In all embodiments, the target and solid phase must be capable of being washed during an assay, without significant loss of the target. This removes unreacted reagents or reaction products that might otherwise interfere with subsequent steps in the assay or the detection.

[0164] In a specific embodiment utilizing polypeptide phosphatases, phosphatase activities to be measured are added as cell lysates, in serum or in other biological fluids, to wells of a microtiter plate where one or more peptide targets has been previously been conjugated. In this specific embodiment, the peptide target is one known to be specific for a phosphatase or is a general substrate for many different phosphatases.

[0165] In various embodiments of this invention which employ Type A Nucleation Centers, following an appropriate incubation period during which one or more phosphatase enzymes, if present, can catalyze de-phosphorylation, de-phosphoramination or de-

phosphorothiolation of the target on the solid phase, the appropriate hetero-bifunctional linkers of the present invention, capable of covalently reacting with phosphoryl, phosphoroamino or phosphorothioate groups on the target, are mixed with the peptide, lipid or oligonucleotide targets and react with the latter if they have been de-phosphorylated, de-phosphoroaminated or de-phosphorothiolated by the phosphatase. At their opposite ends, each of the linkers is a component of a preformed Type A Nucleation Centers. In one embodiment, the enzymes used in fluorogenic detection are also used to create the Nucleation Centers. By using such preformed Type A Nucleation Centers, the rate at which the enzyme is bound to any remaining phosphorylated, phosphoraminated or phosphorothiolated targets is substantially increased. The enzymes used in creating the Type A Nucleation Centers include, but are not limited to, alkaline phosphatase, β -galactosidase, β -glucuronidase, β -lactamase or horse-radish peroxidase.

[0166] In yet a further embodiment using the Type A Nucleation Centers, after a defined reaction period, the plate is washed to remove unreacted nucleation centers and a fluorogenic enzyme substrate, specific for the enzyme, is added. After an appropriate time interval, the activity of the phosphatase is measured.

[0167] In various embodiments of the invention which employ the Type B Nucleation Centers, following an appropriate incubation period during which one or more phosphatase enzymes can catalyze de-phosphorylation, de-phosphoramination or de-phosphorothiolation of the target. The appropriate hetero-bifunctional linkers capable of covalently reacting with phosphoryl, phosphoroamino or phosphorothioate groups on the target are mixed with the peptide, lipid or oligonucleotide targets that are bound to the solid phase to react with the latter if they have been de-phosphorylated, de-phosphoroaminated or de-phosphorothiolated by the phosphatase. At their opposite ends, each of the linkers bears a bioconjugating reagent. This bioconjugating reagent such as biotin, chelating divalent electrostatic linkers such as Nickel, (Histidine)₆, phenylboronic acid, salicylhydroxamic acid, or any of a number of lectins is unreactive with the phosphoramidate, phosphoraminate or phosphorothioate group. In another embodiment, the Type B Nucleation Centers are preformed by covalent combination of an enzyme with one or more such bioconjugation reagents. By such multiplex formation, the rate at which the enzyme is bound to covalently attached hetero-bifunctional linkers is substantially increased.

[0168] In further embodiments, after a defined reaction period involving the linkers and target, the plate is washed, and the corresponding Type B Nucleation Centers added and

allowed to react with the free end of any bound hetero-bifunctional linker. In a variation of this embodiment, the Type B Nucleation Centers can be pre-formed and reacted with the hetero-bifunctional linkers to create Nucleation Centers that are a hybrid of the Type A and Type B Nucleation Centers. However, in either embodiment, once the reaction(s) between the hetero-bifunctional linkers, targets and the Nucleation Centers is complete, the solid-phase is washed to remove unreacted reagents after which a specific fluorogenic substrate for the enzyme is added to the sample, and appropriate time given before detection. In a specific embodiment using the Type 10 Nucleation Centers, the hetero-bifunctional linker is biotinylated at one end and the corresponding complement is an avidin-enzyme conjugate such as avidin-alkaline phosphatase, avidin β -galactosidase, or avidin- β -galactosidase, avidin- β -glucuronidase, avidin- β -lactamase or avidin-horse-radish peroxidase.

Heterogeneous Assays Used in Microtiter Formats

[0169] In yet another general embodiment of the invention the phosphoramidate or phosphoramine chemistry is used for detecting phosphorylation of peptide substrates by one or more specific kinase or phosphatase enzyme(s) in cells, cell lysates or other biological fluids. In one embodiment, the assays are performed in microtiter plate formats and the phosphoramidate or phosphoramine linkers are in solid phase, heterogeneous assays. This method comprises a reaction mixture containing a peptide substrate for one or more kinase or phosphatase enzymes that has: (i) its internal -NH_2 moieties chemically blocked (see, e.g., R'' in Figure 5), and (ii) either the terminal -NH_2 or the terminal -COOH group, being chemically blocked (see, e.g., R' in Figure 5) wherein the unblocked group is covalently attached to a solid phase, such as the thermoplastic of a microtiter plate well (see, e.g., Figure 4b).

[0170] The substrates may be kinase or phosphatase specific targets or they may be generic for many kinases or phosphatases. These substrate(s) are phosphorylated or de-phosphorylated by the actions of a kinase or phosphatase enzyme, respectively, after which they are reacted with a phosphoramidate or phosphoramine linker carrying at least one second conjugating chemistry group, that is not reactive with amine groups, at its opposite end. At any time before, during or after the phosphoramidate or phosphoramine conjugation, a nucleation and detection reagent is reacted with the conjugation chemistry at the linker's second terminus.

[0171] In another embodiment of the invention, the hetero-bifunctional linker may be biotinylated at the end opposite that bearing the phosphoramidite or phosphoramine chemistry and reacted with a streptavidin-enzyme conjugate, in which the enzyme is a highly processive and specific enzyme such as alkaline phosphatase or beta-lactamase, to form a multivalent enzymatically active nucleation center.

[0172] In a further embodiment, following a second washing of the plate to remove unreacted reagents (see, e.g., Figure 4a), a fluorogenic substrate for the streptavidin conjugated enzyme is added to the plate and the plate is incubated for a prescribed period of time. After incubation, the fluorescence intensity of the sample is measured to quantitate the amount of the peptide substrate that is phosphorylated, which is used as a measure of the activity of the kinase or phosphatase enzyme.

Heterogeneous Assays Used in Microtiter Arrays

[0173] In yet another general embodiment of the invention, the phosphoramidate or phosphoramine chemistry of the present invention is used for detecting phosphorylation of peptide substrates by a panel ranging from one to more than 500 different specific kinase or phosphatase enzymes in cell lysates using assays performed in microtiter plate formats using phosphoramidate and phosphoramine linkers in solid phase, heterogeneous assays. This method comprises a reaction mixture containing a peptide substrate for one or more kinase or phosphatase enzymes that has (i) its internal -NH_2 moieties chemically blocked (see, e.g., R'' in Figure 5), and (ii) either the terminal -NH_2 or the terminal -COOH group, being chemically blocked (see, e.g., R' in Figure 5) wherein the unblocked group is covalently attached to a solid phase such as the thermoplastic of a microtiter plate well (see, e.g., Figure 4b).

[0174] The substrates may be selected from a panel of from one to more than 500 kinase or phosphatase specific peptide, lipid or oligonucleotide substrates or, alternatively, they may be one or more generic substrates for many different peptide, lipid or oligonucleotide kinases or phosphatases (separated by target class). In a further embodiment, the method comprises a reaction protocol in which peptide, lipid or oligonucleotide substrates, are covalently attached to a solid phase, such as a well of a microwell plate or a strip of micro wells, within which the substrate(s) are phosphorylated or dephosphorylated by the actions of kinase or phosphatase enzymes, respectively. In these embodiments, the product is then reacted with a phosphoramidate or phosphoramine linker carrying a second conjugating chemistry group at its opposite end that is already a component in a nucleation center. At any

time before, during or after the phosphoramidate or phosphoramine conjugation, a detection reagent is reacted with the conjugation chemistry at the linker's second terminus.

[0175] In a specific embodiment of the invention, the bifunctional linker may be biotinylated at the end opposite that bearing the phosphoramidite chemistry and reacted with streptavidin-enzyme conjugate such as the streptavidin-alkaline phosphatase (see, e.g., Figure 4b) to form the enzymatically active nucleation center. In a further embodiment, following washing of the plate to remove un-reacted reagents, a fluorogenic substrate for the enzyme is added to the plate and the fluorescence intensity of the wells is measured to quantitate the amount of the peptide substrate which is phosphorylated in each. These measurements are then used to profile and compare activities of the different kinase or phosphatase enzymes on one or more peptide substrates in the same microtiter plate.

Heterogeneous Assays Used in Microfluidic Devices

[0176] In yet another embodiment of the invention the hetero-bifunctional linkers and the phosphoramidate and phosphoramine chemistry is used for detecting phosphorylation of peptide, lipid or oligonucleotide substrates by a panel ranging from one to more than 500 different specific kinase or phosphatase enzymes in cell lysates using assays performed in microfluidic device formats and utilizing the phosphoramidate and phosphoramine linkers in fluid phase, heterogeneous assays. In one embodiment, the method comprises a reaction mixture containing a peptide substrate for one or more kinase or phosphatase enzymes that has (i) its internal $-NH_2$ moieties chemically blocked (see, e.g., R'' in Figure 5), and (ii) either the terminal $-NH_2$ or the terminal $-COOH$ group, being chemically blocked (see, e.g., R' in Figure 5) wherein the unblocked group is covalently attached to a solid phase such as the thermoplastic of a microtiter plate well (see, e.g., Figure 4b).

[0177] The substrates may be selected from a panel of from one to more than 500 kinase or phosphatase specific peptide substrates or they may be one or more generic substrates for many different peptide, lipid or oligonucleotide kinases or phosphatases. In a further embodiment, the assays comprise reaction protocols in which substrate, enzyme, hetero-bifunctional linker and the detection reagents are sequestered in different chambers of a microfluidic device, with some fraction from each chamber being transferred through prescribed channels for use in the heterogeneous assay.

[0178] In yet another embodiment, the peptide substrate is first transferred through a channel to a junction where it is mixed with a kinase or phosphatase enzyme and phosphorylated or dephosphorylated, after which the reaction product is moved to another

region of the channel, or another channel, where it is reacted with a hetero-bifunctional linker using the phosphoramidate or phosphoramine chemistry and carrying a second conjugating chemistry group at its opposite end. The product of this reaction is designated the "intermediate reaction product" and can be analyzed using detection methods known in the art. At any time before, during or after the phosphoramidate or phosphoramine conjugation, an appropriate detection reagent can be reacted with the conjugation chemistry at the hetero-bifunctional linker's second terminus. In a specific embodiment, the hetero-bifunctional linker may be biotinylated at the end opposite the $-NH_2$ terminus, and at any time before or during covalent attachment of the linker, but not after, the biotin is allowed to react with avidin to form the nucleation and detection reagent.

[0179] In an alternate embodiment, the large molecular weight component, such as avidin, is allowed to react with the hetero-bifunctional linker before reaction with the peptide substrate to form the nucleation center and the intermediate reaction product is subsequently transferred through the channel, or another channel in the same device, to a region in which the reaction product can be analyzed by fluorescence polarization analysis. In another alternate embodiment, the biotinylated intermediate reaction product can be reacted with a streptavidin-enzyme conjugate followed by subsequent separation of the avidinylated peptide reaction product through the channel, or another channel in the same device, to a region in which the reaction product can be mixed with a fluorogenic substrate and the fluorogenic signal measured to quantitate the amount of the peptide substrate which is phosphorylated.

Solid Phase Heterogeneous Phosphorothioate Assays Used in Microtiter

Formats

[0180] In a further general embodiment of the invention, the hetero-bifunctional phosphorothiolating reagents and linker chemistries are used for detecting phosphorothiolation or dephosphorothiolation of substrates by one or more specific kinase or phosphatase enzyme(s) in cell lysates or other biological fluids in solid phase, heterogeneous microwell-based assays. The substrates may be kinase or phosphatase specific peptides, lipids or oligonucleotides or they may be generic for many kinases or phosphatases within a given structural class. In one embodiment, the method comprises a reaction protocol in which peptide, lipid or oligonucleotide substrates are covalently attached to a solid phase such as a bead or the thermoplastic of the well of a microtiter plate. Within the wells, the substrate(s) are phosphorothiolated or de-phosphorothiolated by the actions of kinase or phosphatase enzymes. The substrates are then reacted with a hetero-bifunctional

phosphorothioate linker carrying a second conjugating chemistry group at its opposite end. At any time before, during or after the hetero-bifunctional phosphorothioate linker conjugation, a detection reagent is reacted with the conjugation chemistry at the linker's second terminus.

[0181] In a specific embodiment, the bifunctional linker is biotinylated at the end opposite that bearing the phosphorothioate linker chemistry and after the linker conjugation, the biotin is allowed to react with a streptavidin-enzyme conjugate. Following washing of the plate to remove un-reacted reagents, a fluorogenic substrate for the enzyme is added to the plate and the fluorescence intensity of the well is measured to quantitate the amount of the peptide substrate which is phosphorylated, which is used as a measure of the activity of the kinase or phosphatase enzyme.

Solid Phase Heterogeneous Phosphorothioate Assays Used in Microtiter

Arrays

[0182] In another general embodiment of the invention, hetero-bifunctional phosphorothiolation reagents and linker chemistries are used for detecting phosphorothiolation or de-phosphorothiolation of substrates by kinase or phosphatase enzymes in cell lysates or other biological fluids in solid phase, heterogeneous arrays. In one embodiment, the method comprises a reaction mixture containing a peptide, lipid or oligonucleotide substrate for one or more kinase or phosphatase enzyme(s) that has (i) its internal $-NH_2$ moieties chemically blocked (such as R'' in Figure 1), and (ii) either the terminal $-NH_2$ or the terminal $-COOH$ group, being chemically blocked by a method known in the art (see, e.g., R' groups in Figure 1) with the unblocked group being covalently attached to a solid support such as the thermoplastic of a microtiter plate well (Figure 4). The substrates may range from one to more than 500 kinase or phosphatase specific peptides, lipids or oligonucleotides or they may be one or more generic substrates for many kinases or phosphatases.

[0183] In a further embodiment, the method comprises a reaction protocol in which peptide, lipid or oligonucleotide substrates are covalently attached to a solid phase, such as a well of a microtiter plate or a bead, on which the substrate(s) are subsequently phosphorothiolated or de-phosphorothiolated by the actions of kinase or phosphatase enzymes. Once phosphorylated or de-phosphorylated, the target substrates are reacted with a hetero-bifunctional phosphorothioate linker carrying a second conjugating chemistry group at its opposite end. At any time before, during or after the hetero-bifunctional phosphorothioate

linker conjugation, a detection reagent is reacted with the conjugation chemistry at the linker's second terminus.

[0184] In a specific embodiment, the bifunctional linker may be biotinylated at the end opposite that bearing the phosphorothioate linker chemistry and, after incubation, the biotin is reacted with streptavidin-enzyme conjugate. Following washing of the plate to remove unreacted reagents, a fluorogenic substrate for the enzyme is added to the plate and the fluorescence intensity of the well is measured to quantitate the amount of the peptide substrate which is phosphorylated. This measurement is used to profile and compare activities of the different kinase or phosphatase enzymes on one or more peptide substrates in the same microtiter plate.

Heterogeneous Phosphorothioate Assays Used in Microfluidic Devices

[0185] In yet another general embodiment of the invention, the hetero-bifunctional linkers and the phosphorothiolation reagents and chemistries are used for detecting phosphorothiolation of peptide, lipid or oligonucleotide substrates by a panel of from one to more than 500 different specific kinase or phosphatase enzymes in cell lysates using assays performed in microfluidic device formats using phosphorothioate linkers in fluid phase, heterogeneous assays.

[0186] In one embodiment, the method comprises a reaction mixture containing a peptide, lipid or oligonucleotide substrate, which may be fluorescent labeled depending upon the detection method to be used, for one or more kinase or phosphatase enzyme(s) that has its internal $-NH_2$ moieties, terminal $-NH_2$ and $-COOH$ groups chemically blocked by such methods as are known to one skilled in the art (see, e.g., R' and R" in Figure 1). The substrates may be selected from a panel of from one to more than 500 kinase or phosphatase specific peptide, lipid or oligonucleotide substrates or they may be one or more generic substrates for many different kinases or phosphatases.

[0187] In a further embodiment, the assays comprise reaction protocols in which the peptide substrates, enzyme, hetero-bifunctional linker and the detection reagents are sequestered in different chambers of a microfluidic device, with some fraction from each chamber being transferred through prescribed channels for use in the heterogeneous assay. In the first phase of the assay, the peptide substrate is transferred through a channel to a junction at which it is mixed with a kinase or phosphatase enzyme and phosphorothiolated or dephosphorothiolated, after which the reaction product is moved to another region of the channel, or another channel. There, it is reacted with a hetero-bifunctional linker using the

phosphorothioate chemistry and carrying a second conjugating chemistry group at its opposite end. The product of this reaction is designated the "intermediate reaction product" and can be analyzed by methods known in the art. At any time before, during or after the phosphorothioate conjugation, an appropriate detection reagent can be reacted with the conjugation chemistry at the hetero-bifunctional linker's second terminus.

[0188] In a specific embodiment, the hetero-bifunctional linker may be biotinylated at the end opposite the $-NH_2$ terminus, and at any time before or during the reaction leading to the covalent linkage of the phosphorothioate linker, the biotin is allowed to react with avidin as the detection reagent. In an alternate embodiment, the avidin is allowed to react with the hetero-bifunctional linker before reaction with the substrate and the intermediate reaction product is subsequently transferred through the channel, or another channel in the same device, to a region in which the reaction product can be analyzed by fluorescence polarization analysis. In yet another alternate embodiment, the biotinylated intermediate reaction product can be reacted with a streptavidin-enzyme conjugate followed by subsequent separation of the avidinylated substrate reaction product through the channel, or another channel in the same device, to a region in which the reaction product can be mixed with a fluorogenic substrate and the fluorogenic signal measured to quantitate the amount of the peptide substrate which is phosphorylated.

Fluorescence Polarization Analytical Methods

[0189] In one general embodiment, the homogeneous assays are binding assays in which a small fluorescent peptide or oligonucleotide reagent, having a fast rotational correlation time, is covalently bound to a much larger detection reagent, which has a much slower rotational correlation time. This has a corresponding effect on the extent to which a fluorescent solution that is excited using plane polarized light, also emits in the plane of excitation or remains polarized. In a specific embodiment, the linker conjugates provide much higher values of mP than the unreacted fluorescent peptides and oligonucleotides in the kinase and phosphatase assays.

[0190] In another embodiment of the present invention, a fluorescent labeled peptide or oligonucleotide is used as a reagent. Examples of these reagents include phosphorylatable or phosphorothiolatable peptide substrates for kinase enzymes such as the serine/threonine and tyrosine kinases and the corresponding phosphatases. In a further embodiment, nucleation reagents such as conjugates of hetero-bifunctional linkers with oligomeric polymers such as polyamino acids, polypeptides, synthetic polymers such as polylysine, poly acrylic acid,

polyamines and polysulfones, and mixed co-polymer derivatives such as the polyamine derivatives of polyoxyethylene-polyethylene oxide are used.

[0191] In another embodiment, the molecular mass of the high molecular weight reagent ranges in size from about 5000 daltons to greater than 100,000 daltons. In a further embodiment, the molecular mass of the high molecular weight reagent ranges in size from about 10,000 to 100,000 daltons.

Homogeneous and Heterogeneous Kits

[0192] One general type of kit is a homogeneous fluorescence polarization kit for microtiter plate based assays of kinase or phosphatase activity. The kit includes a microtiter plate; a known amount of a fluorescent peptide, lipid or oligonucleotide target; ATP or an ATP structural analog; a hetero-bifunctional phosphoramidite, phosphoroamino or phosphorothioate linker; purified avidin or another high molecular weight reagent such as casein; and a purified enzyme, whether naturally derived or recombinant. In further embodiments, instructions for determining the fluorescence polarization of the substrate, mixing the high molecular weight and hetero-bifunctional linkers prior to the assay to form nucleation centers, and determining the fluorescence polarization of the mixture and comparing that value to the P value obtained for the starting substrate are provided.

Homogeneous Assay Kit Configuration

[0193] In general embodiments of the invention, the Homogeneous Assay Kit Configurations include assays that are performed in the following devices: (i) microtiter plates and fluorescence microwell plate readers, (2) microfluidic devices, (3) bead-based separatory devices, (3) fluorescence microscopes, and (4) electrophoresis gels. In the preferred assay formats, peptide targets are typically from 5 to 50 amino acids in length and have relatively fast rotational diffusion rates. The fluorescent label may be any derivative of the commercially available fluorescent dyes such as fluorescein, Texas Red or the Alexa dyes or they may have the large Stoke's shifts and stability against photobleaching of the StarBright® labels.

[0194] In one embodiment of the homogeneous microtiter plate assay, each well contains 5 µl of an initial Kinase Reaction Mixture. In a specific homogeneous assay for the alpha isoform of Protein Kinase C (PKC-alpha), the PKC Reaction Mixture is drawn from a stock prepared in the ratios shown:

- 1530 ul PKC Alpha Reaction Buffer
- 450 ul Lipid Reagent
- 225 ul PKC Alpha Peptide Reagent
 - 45 ul PKC Alpha Enzyme

[0195] In this embodiment, 5 ul of an ATP or ATP analog solution is added to each well after which the reaction mix is covered and incubated at room temperature in the dark for 90 minutes. At this time, 20 ul of Nucleation Reaction Mixture containing either the phosphoramidite, the phosphoroamino or the phosphorothioate nucleation centers is added to each well of the microwell plate which is again covered and incubated at room temperature (21-25°C) in the dark for two hours. The final product is then analyzed. A variety of known detection schemes may then be employed to detect changes in the rotation, lateral translation or molecular mass of a molecule such as NMR, ESR, phosphorescence, fluorescence correlation spectroscopy and spin resonance exchange.

[0196] In a further embodiment, fluorescence polarization data is developed as the ratio of (1) the difference of the parallel and perpendicular fluorescence emissions to (2) the sum of these two emissions and are independent of the fluorescence intensity itself. The polarization value can be compared to known standards for which the P values of peptide or oligonucleotide standards which are either completely unreacted or completely reacted with multiplexed nucleation centers. In an alternate embodiment, the P values can be determined pre and post reaction and the difference between the two values used as a measure of the amount of final product produced. Using either method of measurement, the P value serves as a measure of the extent of the reaction of interest by indicating the amount of final product produced.

[0197] In yet another embodiment, the homogeneous protein kinase assay methods can be carried out in a microfluidic device which contains at least one channel and one or more reservoirs (also called "chambers") within the body of the device. Channels and reservoirs may be separate and discrete and can be connected via fluids in the channels, at channel intersections including cross intersections, "T" intersections or multiply branched connections within which fluid subsamples can be moved, mixed or separated.

[0198] In a specific example, the microfluidic device has a structure that includes a network of internal channels that are connected to a series of reservoirs within which the reagent solutions are distributed. These reservoirs may be used to store and then introduce

various reagents into the channels of the device and ultimately into a detection channel or reservoir where the final products are analyzed.

[0199] The device may, in further embodiments, also depend upon the use of any of a number of ancillary elements used for introducing reagents into the wells such as capillary tubes, electrospeoting dispensers, syringe injectors and manual or automated pipettors. The assays performed in such systems typically rely on pressure, vacuum, electrophoresis or electrokinetic material transport and direction to move reagents through a channel and between reservoirs. A variety of detection schemes may be employed which detect changes in the rate of rotation or lateral diffusion of a molecule that result from changes in its molecular size, mass or volume. Examples of such detection methods range from electrophoretic sieving to several of the same spectroscopic techniques described for microtiter configurations above. In specific embodiments, the use of changes in fluorescence polarization are used.

[0200] In a specific embodiment comprising PKC-alpha, the reaction mixture consists of: PKC Alpha Reaction Buffer; Lipid Reagent; PKC Alpha Peptide Reagent; PKC Alpha Enzyme; and one to 1000 nanoliters of the mixture in reservoir 2 of the device. A small volume of ATP or ATP analog solution is then transferred electroosmotically to a T-junction and the reaction mix is held in position at the junction for anywhere from 0.1 to 10 minutes, after which the volume at the junction is moved to a second T junction where it is mixed with the appropriate volume of a preformed synthetic nucleation center mixture from a third reservoir containing either the phosphoramidate, the phosphoramino or the phosphorothioate nucleation centers. This second reaction mix is also incubated for from 0.1 to 10 minutes and then the final product is analyzed by fluorescence polarization. In such a device, an optical window may be positioned across one or more channels or reservoirs of the device which can transmit both exciting and emitted light. In specific embodiments, the homogeneous assays typically rely upon a change in the level of fluorescence polarization of the final product and an appropriate set of polarizing filters is used to separately detect emissions that are parallel and perpendicular to the plane of the exciting light.

[0201] The following specific examples are provided solely to illustrate particular representative embodiments of the invention. Accordingly, the following examples should not be construed as limiting the scope of the invention in any way.

EXPERIMENTAL PROCEDURES

[0202] Materials: Fluorescence excitation and emission spectra were measured on a Hitachi F-4500 fluorescence spectrophotometer in ratio mode with a rhodamine B quantum counter. For fluorescence polarization experiments, two different detection instruments were used, a BMG Polarstar and an LJL Analyst. Molecular brightness measurements in 96-well and 384-well microtiter plates were performed in the following four instruments: (1) the LJL analyst, (2) the Tecan Ultra, (3) the PerSeptive BioSystems Cytofluor II, and (4) Chromagen's own photon counting instrument as described in US Patent Application Serial No. 10/039,769.

[0203] In general, the hetero-bifunctional linkers of the present invention are of the general formulae shown in Figure 1. StarBright® dyes in the form of either isothiocyanate or N-hydroxysuccinimide esters were prepared as described in U.S. Provisional Patent Application No. 60/413,025. 6-(fluorescein-5-carboxamido)-hexanoic acid succinimidyl ester, single isomer (F-NHS) was purchased from Molecular Probes. Dimethylformamide (DMF), acetonitrile (HPLC Grade), trifluoroacetic acid (TFA), 6-aminohexane, Carbodiimide, sodium phosphate, mono- and dibasic, were all of reagent grades and were purchased from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimidyl sulfonate (sulfo-NHS), and sulfo-NHS-methylacetate (sulfo-NHS-acetate) were purchased from Pierce. Avidin was purchased from Calbiochem. Protein Kinase C isoforms was purchased from Panvera, Madison, Wisconsin, or Sigma – Aldrich and were used following the vendor protocol in 20 mM HEPES, 10 mM MgCl₂, 100 μM CaCl₂, 100 μg/ml phosphatidylserine (Sigma), 20 μg/ml dioleoylglycerol (Sigma), and 0.03% Triton X-100, pH 7.4. Adenosine-5'-triphosphate (ATP) and Adenosine -5'-O-(3-thiophosphate) were purchased from Sigma. EZ-Link Biotin -LC-Amine, maleimide-biotin BMCC, iodoacetyl-LC-biotin and LC-SMCC were products of Pierce. In addition to the use of fluorescence polarization, kinase and phosphatase assays were also analyzed using Maldi MS analysis performed on PerSeptive Biosystem Voyager-DE using alfa-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. HPLC purification was performed on BioCad using Vydec C18 column. Calf intestinal alkaline phosphatase (AP) was purchased from Roche; streptavidin was purchased from Calbiochem.

Example #1--Protection of Non-target Amino and Hydroxyl Groups on Kinase Targets, Labeling of Targets Prior to Use With the Hetero-bifunctional Linkers (Figure 5)

[0204] In general it has proven unnecessary to protect non-target hydroxyl groups and other moieties on oligonucleotide targets and phospholipid targets. By contrast, peptide

targets are generally appropriately protected before use of the linker chemistry. Specifically, to avoid the possibility that terminal carboxyl groups and pendant hydroxyl or amino groups of amino acids in peptide targets might also react with the phosphoramidate moiety of the present invention, peptide targets were first fluorescent labeled and then any pendant amino and hydroxyl groups were protected prior to use of the peptide substrate in protein kinase or protein phosphatase assays.

[0205] The protection of the peptide substrate sequence, RFARKGSLRQKNV, a generic substrate for the isozymes of Protein Kinase C, is used as a representative illustration of the labeling and protection procedures used for peptide substrates in general. The substrate was first fluorescent-labeled at the amino terminus after which the ϵ -amino groups of the internal lysines and the terminal $-\text{COOH}$ group were sequentially protected.

Fluorescent Labeling of the PKC Peptide, RFARKGSLRQKNV (Figure 5, peptide 2)

[0206] The PKC peptide 1 (2.028 mg, 1300 nmols) was dissolved in 0.1M phosphate buffer (150 μl), pH 7.2, and treated either with fluorescein-NHS or StarBright® Dye Isothiocyanate in DMF (84.50 μl of 20 mM solution of fluorophore in DMF, 1690 nmols) at room temperature for 12-16 hours. Maldi MS analysis of the reaction mixture usually showed 90-95% labeling. The reaction mixture was then concentrated to dryness under vacuum of Savant Speed-Vac. The resulting residue was suspended in 10% acetonitrile/water, vortexed and centrifuged to remove the soluble peptide and purified by HPLC on a C18 column using a linear gradient of 10 acetonitrile in 0.1% TFA to 100% acetonitrile in 0.1% TFA over a period of 30 minutes at a flow rate of 1.5 ml/min, collecting about 2 ml each fractions. Each of the fractions was analyzed by Maldi MS and the fractions primarily giving the labeled peptide 2 (m/e 2032) were concentrated to dryness to yield the labeled peptide 2 in 40 to 60% yield.

Protection of Lysine Amines (Figure 5, peptide 3)

[0207] F-PKC, 2 (0.2 mg, 100 nmols) was dissolved in 0.1M phosphate buffer pH 7.2 (7.5 μl) and DMF (7.5 μl added), followed by the addition of 100 mM sulfo-NHS acetate in DMF (2.5 μl , 250 nmols). The reaction vial was gently shaken at room temperature (RT) for 4 hours and then analyzed by Maldi MS which showed almost quantitative diacetylation to yield F-PKC-(Lysine-NHAc)₂, the desired peptide 3, m/e 2119 (calc.: 2118)

Protection of C-Terminal Carboxylic Acid Group to Produce F-PKC-(Lysine-NHAc)2-CONH-R) (Figure 5, peptide 4)

[0208] To the above crude reaction was added sulfo-NHS in DMF (5 ul of 200 mM solution) followed by the addition of EDC (10 ul of 200 mM solution in water), and 6-aminohexane, pH 7.0 (3 ul, 1M solution), and DMF (18 ul). Heated the reaction contents at 50°C for 3 hours at which time Maldi MS showed two ions, almost in 1:1 ratio, at m/e 2203 (calc.: 2202, Figure 8a) for F-PKC-(Lysine-NHAc)2-CONH-Hexane), m/e 2119 due to the unreacted starting material, 3. HPLC purification on a C18 column produced peptide 4 in 30 % yield.

Phosphorylation to Produce Phosphorylated Peptide (Figure 7, compound 5)

[0209] The peptide 4 (1000 pmols) was incubated with ATP (2000 pmols) and PKC-alpha (2 pmol) at 32°C for 1 hour in 20 mM HEPES buffer (10ul) as described by the vendor. Maldi-Mass spectra of the reaction mixture showed an intense ion at m/e 2284 (calc.: 2283 for phosphoryl-peptide 5) (Figure 8b).

Phosphoramidation with EZ-Link Biotin to Produce Biotinylated Peptide (Figure 7, compound 6)

[0210] The phosphorylated peptide 5 (10 nmoles) and carbonyldiimidazole (100 nmoles) were allowed to react in 0.1 M HEPES buffer, pH 7.2, at 37°C for 3 hours. To the reaction mixture was then added EZ-Link-Biotin PEO-LC-Amine (150 nmols) in 0.1M HEPES, pH 7.2. The reaction mixture was then heated at 50°C for 1 hour. Maldi MS analysis (Figure 8c) showed an ion at m/e 2583 that was diagnostic of the formation of phosphoramidate linkage between the amine group of EZ-Link -Biotin and the phosphoryl peptide 5 giving rise to the phosphoramidated peptide 6. Simultaneously, phosphoramidation reaction was carried out on the peptide which was not phosphorylated, for example, F-PKC-(Lysine-NHAc)2-CONH-Hexyl to provide a negative control.

Fluorescence Polarization

[0211] The peptide 6 (50 pmols) and the negative control (50 pmols) were mixed with avidin (25 pmols) at pH 7.0 in 20 mM HEPES buffer, pH 7.0. FP measurements were performed on LJL Analyst. Fluorescence polarization values in the range of 50-100 mP were observed in the preliminary, unoptimized experiments.

Example #2—Phosphoramidate Linkage Chemistry for Covalent Attachment to Hydroxyl Moieties on Phosphoryl Groups

[0212] Phosphorylation of SBG labeled- Phosphatidyl Inositol to Produce Phosphorylated SBG – PI 4,5)(Figure 2b):StarBright® Green- Labeled - Phosphatidyl Inositol (1000 pmpols) was incubated with ATP (2000 pmols) and PKC-alfa (2 pmol) at 32°C for 1 hour in 20 mM HEPES buffer (10ul) as described above. Maldi MS of the reaction mixture showed an intense ion corresponding to the molecular mass of the predicted conjugate. At that time the target was between 0 and 100 % phosphorylated as shown in parallel reactions using radiolabeled ATP.

Example #3—Preformation of Synthetic Nucleation Centers

Synthetic Nucleation Centers for Homogeneous Fluorescence Polarization Assays

[0213] A representative example of the preparation of synthetic reactions centers for the rapid reaction methods of the present invention involves incubation of 2 molar equivalents of biotinylated hetero-bifunctional linker with 1 molar equivalent of avidin in 100 mM HEPES buffer (pH 7.4) for 15 minutes at 37°C. At the end of this time, the reagents are virtually 100% reacted giving rise to a hydrophobically bound avidin-biotin-linker-X complex (the “synthetic nucleation center”), where X=NH₂,S-, maleimido, iodoacetyl, NHS, ester or other conjugation chemistry group known to one skilled in the art. In general, X is a group capable of undergoing covalent conjugation with the phosphoryl group of a target, or, in the case of Chemistries II or III, with the amino or thiol group of a phosphoroaminated or phosphorothiolated target, respectively.

[0214] In a typical homogeneous assay preparation for the PKC-theta assay, two molar equivalents of the synthetic nucleation center were added to a kinase reaction mixture that had also been preformed using either ATP or γ -NH₂-ATP or γ -S-ATP as a donor. The reaction mix was incubated at 37°C for 1 to 2 hours, after which fluorescence measurements were made relative to a negative control in which the kinase reaction was conducted in the presence of 5mM EDTA, an inhibitor of kinase enzyme. Maldi MS and fluorescence polarization were generally used to determine the extent of phosphorylation, phosphoramination or phosphorothiolation of the peptide target.

Synthetic Nucleation Centers of Type A for Heterogeneous Assays

[0215] In general, all of the heterogeneous reaction protocols using either Type 10 A or Type 10 B nucleation centers are fluorogenic. After the reaction with the nucleation centers, the microwell plate is washed to remove unreacted nucleation centers and then a fluorogenic enzyme substrate is added. After one hour the fluorescence is measured and compared to positive standards to determine the extent of phosphorylation, phosphoramination or phosphorothiolation of the target.

[0216] Prior to the actual assay, and in a procedure analogous to that used for pre-formation of the synthetic nucleation centers used in homogeneous assays, nucleation centers of Type 10A are preformed through covalent interactions, as described above, of a hetero-bifunctional linker reagent with an enzyme such as alkaline phosphatase or beta-lactamase. In all cases of conjugation, whether to avidin, streptavidin or to another enzyme or polypeptide, the enzyme component was prepared in a 40 fold excess of the linker in 0.1 M HEPES buffer, pH 7.5, containing 10% DMF. A typical linker that is used is either succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxy-6-amidocaproate (LC-SMCC) or succinimidyl-3-(bromoacetamido)-propionate (SBAP). After gently shaking the reaction mixture on a vortex mixer for one hour, the reaction mixture was de-salted on a G-35 column (1x40 mm) using 0.1 M MOPS buffer, pH 7.0. The polypeptide peak was collected and concentrated on an Amicon Centricon C-30 concentrator to a volume of 500 µL and the final concentration determined by reading the absorbance at 280 nm. In a representative assay, two molar equivalents of the enzyme-based nucleation center were added to a microwell plate containing phosphorylated, phosphoramined or phosphorothiolated peptide target that had previously been treated with cell lysates as described above and allowed to react at 37°C for two hours. At the end of this time, the microwell plate was washed and scanned to establish the baseline luminescence after which the fluorogenic substrate was added, the plate incubated again for one hour at 37°C and the fluorescence of the plate measured again. After the reaction with the nucleation centers, the plate was washed to remove unreacted centers and a fluorogenic substrate added. After one hour the fluorescence was measured and compared to positive standards to determine the extent of phosphorylation, phosphoramination or phosphorothiolation of the target.

Synthetic Nucleation Centers of Type B for Heterogeneous Assays

[0217] Synthetic Nucleation Centers of Type 10 B for heterogeneous assays avidin is, by itself, multi-valent. In a procedure analogous to that used for pre-formation of the synthetic

nucleation centers of Type 10 A, nucleation centers of Type 10B were preformed as described above except that the avidin reagent was covalently bound to an enzyme such as alkaline phosphatase or beta-lactamase and the product used directly as the nucleation center in reaction with biotinylated hetero-bifunctional linkers after they had been reacted with target. As before, the avidin was first activated by the addition of 20 molar equivalents of hetero-bifunctional linker and then 20 molar equivalents of the appropriate enzyme were added at 37°C. After four hours, the avidin-enzyme conjugates were separated and purified from the reaction mix using a Centricon-100 and the conjugation ratio determined using Maldi MS to be 1 to 2 moles of avidin:1mole enzyme.

[0218] In a typical reaction, the enzyme-avidin conjugate is used in a 2:1 molar excess to the maximum amount of target, together with a fluorogenic substrate as described herein, to create the fluorogenic heterogeneous assays of the present invention. After the reaction with the nucleation centers, the plate was washed to remove unreacted centers and a fluorogenic substrate added. After one hour the fluorescence was measured and compared to positive standards to determine the extent of phosphorylation, phosphoramination or phosphorothiolation of the target. In a typical preparation for the PKC-theta assay, the avidin enzyme conjugates were mixed with biotinylated hetero-bifunctional reagent in a mole ratio of 1 mole enzyme-avidin to 2 moles of biotinylated hetero-bifunctional linker in modified MES buffer and gently stirred for 15 minutes at 37°C. At the end of this time, the reagents are virtually 100% reacted were purified by HPLC chromatography for further analysis.

[0219] In a typical preparation such as one for the heterogeneous PKC-theta assay, the avidin enzyme complex and the biotinylated hetero-bifunctional linkers were mixed in a mole ratio of 1:2 in TAPS buffer and gently stirred for 15 minutes at 37°C. At the end of this time, the reagents are virtually 100% reacted as shown in the Maldi MS and consist of multivalent nucleation centers capable of linking targets that are phosphorylated, phosphoramined or phosphorothiolated to the enzyme conjugate. In a typical reaction, the enzyme-avidin conjugate is used in a 10:1 molar excess to the maximum amount of target, together with a fluorogenic substrate as described herein, to create the fluorogenic heterogeneous assays of the present invention

Example 4--Phosphoroamino Chemistry II Phosphoroamination Linkage Chemistry for Covalent Attachment to Amino Moieties on Phosphoroamino Groups

N-benzoyl-adenosine-2'-(tert-butyl-dimethylsilyl)-5'—(p-chlorophenyl-3-cyanoethyl)-phospho- ryl-3'-succinylethylacetamido-Long Chain Alkyl-CPG (Figure 11)

[0220] In a 100 ml round bottom flask fitted with a fritted disc filter (medium size) and a Teflon stopcock was taken the commercially available N-benzoyl-adenosine loaded CPG8 (Figure 11, compound 8) (3 gms, 100 umols) and was gently shaken with 3% trichloroacetic in dichloromethane (25 ml) for 5 minutes at RT. The resin was then filtered under vacuum and washed successively with dry CH_2Cl_2 (4X20 ml) and dry pyridine (2X20ml). After the final wash, the resin was dried by under vacuum for 10 minutes to give the detritylated CPG 9 with its 5'-OH free (Figure 11, compound 9). The resin 9 was then suspended in pyridine (30 ml) and reacted with p-chlorophenyl-(cyanoethyl-chloro)-phosphate (Figure 11, compound 10) (300 umols) at RT for 1 hour. At the end of this time, the reaction mixture was filtered and washed successively with dichloromethane and pyridine as described above to remove the unreacted compound resulting in the phosphoryl-protected-CPG 11 (Figure 11, compound 11).

N-benzoyl-adenosine-5'-(p-chlorophenyl-diphosphate)- CPG 14

[0221] The resin-bound product 11 was treated with a mixture of 3:1:1 pyridine/triethylamine/water (100 ml) for 2 hours at RT to remove the cyanoethyl-group to yield 12. The resin was then filtered and washed with pyridine (5X30 ml) and then dried under vacuum. The compound 10 (300 umol) in pyridine (30 ml) was added to de-cyanoethylated resin 12 and the reaction vessel was gently shaken for 2 hours at RT. At the end of this period, the reaction mixture was filtered, and washed with CH_2Cl_2 (4X30 ml) and pyridine (2X20 ml) and then dried under vacuum to give the protected diphosphate 13 (Figure 11, compound 13).

N-benzoyl-adenosine-2'-(tert-butyl-dimethyl-silyl)-3'-(succinyl-acetamido-LC-CPG)-5'- α -(p-chlorophenyl)-phosphoryl- β -(p-chlorophenyl)-phosphoryl- γ -(amino)-phosphate

[0222] The resin 13 was decyanoethylated using a mixture of pyridine-treithylamine and water as described above. The resulting resin-bound diphosphate 14, after drying under vacuum, was treated with commercially available compound 15 (500 umol) in pyridine (30 ml) at RT for 2 hours. The resin was filtered and reacted with water (20 ml) for 30 minutes to obtain the hydrogen-phosphonate 16 (Figure 11, compound 16). After 30 minutes, the resin was filtered, and washed with pyridine (3X20 ml). It was then suspended in pyridine (30 ml) and NH_3 gas bubbled for 1 hour. The reaction mixture was then filtered and washed with CH_2Cl_2 (4X40 ml) to accomplish the formation of the desired γ -amino-protected ATP, still bound to the resin (Figure 11, compound 17).

Deprotection and Purification to Obtain γ -Amino-ATP

[0223] Resin 17 was transferred into two high pressure screw-cap glass tubes and treated with 0.2 M tetramethyl-guanidium solution in pyridine (10 ml each) for four hours at RT to cleave the p-chloro-phenyl protecting group from the phosphate groups of the triphosphate 17. After four hours, the supernatant was removed and discarded. The resin was treated with ammonium hydroxide (28%, 8 ml each) at 60°C for 6 hours to cleave the product from CPG and to deprotect the benzoyl group from adenosine. At the end of this treatment, the supernatant was collected, the resin washed twice with water (4 ml each) and the combined solution was concentrated on speedvac to dryness. The dried product was treated with 1.0 M tetrabutylammonium fluoride/ THF (TBAF) (7 ml each) for two days or until the HPLC showed near complete removal of tert-butyldimethyl silyl group. The solvent, THF, was removed under vacuum at RT. The residue left was dissolved in 0.1 M triethylammonium acetate (TEAA), pH 7.0, filtered through 0.2 μ M filter and purified on a reverse phase C18 column using a linear gradient of 1.0 % to 50 % acetonitrile in TEAA. The product peak was collected, concentrated to dryness and then desalted on a Sep-Pak column resulting in pure γ -amino-ATP (Figure 11, compound 7). The mass spectrum showed a major ion at m/e 530.60 (calculated mw 531).

Phosphoroamination of a PKC target using γ -amino-ATP (Figure 12).

[0224] Peptide 4 (Figure 12, peptide 4) (1000 pMoles) was incubated with γ -amino-ATP (2000 pMoles) and PKC- α (2 pMoles) in a volume of 10 μ L at 32°C for 1 hour in 20 mMolar HEPES buffer, pH 7.4 containing 132 μ Molar phosphatidyl serine, 32 μ Molar diolylglycerol, 10 mMolar MgCl₂, 10 μ Molar CaCl₂. At the end of this time, the Maldi-Mass spectra of the reaction mixture showed a major ion an m/e 2278 (expected 2279) which was diagnostic of the formation of the phosphoroaminated PKC target (Figure 12, compound 18).

[0225] Compound 18 was then reacted with LC-NHS-biotin to produce phosphoramidated peptide 19 (Figure 12, compound 19a). The above crude reaction mixture was treated with 1 mL of 50 nMolar EDTA to de-activate PKC- α , followed by treatment with 2 mL of 1 mMolar LC-NHS-biotin in multiplexed Nucleation Centers (2000 pMole) that were preformed by reaction of the multiple hetero-bifunctional linkers with avidin by the method previously described. The mixture was then heated to 37°C for 2 hours. At the end of the reaction time, the analysis by fluorescence polarization showed values of ranging from

100 to 200 mP relative to controls that had been incubated in EDTA or had not been phosphoramidated.

Example 5--Phosphorothioate Linkage Chemistry for Covalent Attachment to Sulfhydryl Moieties on Phosphorothiol Groups

[0226] Some kinase and phosphatase enzymes act upon substrates that have numerous hydroxyl groups in addition to those present on phosphoryl groups which could potentially react with the phosphoramidate moieties of the present invention which could be difficult or laborious to block, as in the case of phospholipids. As an alternative examined application of conjugation chemistries using the structural analog of ATP, adenosine 5'-O-(3-thiotriphosphate) or "ATP γ S". ATP γ S has been used for many years as an alternative to ATP for thiophosphorylating peptides and nucleic acids, however, as reported earlier, the prior art teaches away from the possibility that this analog can be successfully utilized in homogeneous microtiter plate-based assays. Moreover, no prior art exists which demonstrates the use of this analog in heterogeneous microtiter based assays. The new studies of the present invention have discovered novel procedures with which some, but apparently not all, kinase enzymes can utilize this ATP analog in replacement of ATP and enable use of the analog in both homogeneous and heterogeneous assays.

[0227] In the homogeneous assay method of this invention, one or more hetero-bifunctional linker of the general formula shown in figure 13 are mixed in with avidin and the mixture allowed to react on ice for 15 to 45 minutes to form nucleation centers as by a similar method to the one previously described.

[0228] In a specific example, 1 μ g of dry hetero-bifunctional linker, such as maleimide-(CH₂)_n - biotin, where n=1 to more than 20, was first dissolved in 500 μ L of DMSO and mixed well with avidin (typically 2 to 5 molar equivalents) by vortexing for 5 seconds to create the Biotin/Avidin based Nucleation Center which contained the maleimides groups at the outer edge of the center itself. Fifteen to forty-five minutes before use, this nucleation reagent containing the nucleation centers was prepared by combining the following reagents in a 15 mL tube: 7.7 mL Detection Buffer with 440 μ L Biotinylation Reagent and 880 μ L solution of avidin in a stoichiometric ratio ranging from between 4:1 to 10:1.

[0229] In a parallel reaction, 22 μ L of a Kinase Reaction Mixture or a Phosphatase Reaction Mixture was prepared in a 15 mL tube and placed on ice: to 1500 μ L of Kinase Reaction Buffer was added 480 μ L of stock of Lipid Reagent, containing any phospholipids

appropriate for optimum kinase activity, 240 μ L of a stock of fluorescent labeled peptide substrate, such as StarBright® Green-RFARKGSLRQKNV, 190 μ L of a kinase enzyme such as the theta isoform of Protein Kinase C, PKC- θ or a phosphatase enzyme. Separately 2500 μ L of ATP γ S Working Solution was prepared as follows:

Reagent 1: 5 mL, of concentrate 1 of ATP γ S with 495 mL of kinase reaction buffer in a 1.5 mL tube to make a stock ATP γ S stock solution, vortex and keep on ice;

Reagent 2: 100 μ L of the ATP γ S Stock Solution of concentrate 2 was diluted with 2400 μ L of Kinase Reaction Buffer in a 15 mL tube to make the ATP γ S Working Solution, vortexed and kept on ice.

[0230] The kinase or phosphatase reaction and subsequent detection of the extent of phosphorylation was performed as follows:

Stage 1: 5 μ L of the Kinase Reaction Mixture was placed in each well of a 384-well microtiter plate. If any test compound was to be included, 1 μ L of the test compound at the desired concentration was added to each well. In parallel, standards were separately added to the wells used to establish a standard curve. To begin the kinase or phosphatase reactions, 5 μ L of ATP γ S substrate solution were dispensed into each well, the plate was covered to help reduce evaporation and the plate incubated at room temperature (21 to 25°C) for 45 minutes.

Stage 2: dispense 20 μ L of the Nucleation Center Reagent into each well of the microtiter plate and incubated the reaction mix a second time for 1 hr at room temperature (21 to 25°C). At the end of the incubation period, read the assay plate in a fluorescence polarization micro plate reader.

Example 6--General Homogeneous Fluorescence Polarization Assay of Phosphorylation, Phosphoroamination or Phosphorothiolation in Microtiter Plates

[0231] The theta isozyme of Protein Kinase C is used here to represent all kinase and phosphatase enzymes and the homogeneous measurements of their activities using any of the three chemistries. Essentially the only difference between the assays is in whether it is ATP or an ATP analog is that is used as the donor in the case of the kinases, thereby establishing the Chemistry used to conjugate the target with the Nucleation Centers, whereas in the case of the phosphatases, the target is the donor and the determination of the linker chemistry used in attaching to the Nucleation Center is determined by whether the target is phosphorylated, phosphoroaminated or phosphorothiolated. In all cases, purified enzymes or the

corresponding recombinant forms are added to the wells of the microtiter plate and the isozymes, if present, are allowed to act on the target that is dispersed in the liquid phase.

Homogeneous measurement of the activity of PKC

[0232] In the preferred configuration, shallow well microplates having densities or 96 or 384 wells are used. Prior to the preparation of the enzymes and mixing with other reagents, the Nucleation centers are preformed according to a similar method as the one described in Example 3. Similarly, the fluorescent labeled targets were prepared in advance and, if necessary, protected according to a similar method as the one described in Example 2. In the homogeneous assay method of this invention, hetero-bifunctional linkers were used except that the chemistry used to react with the target was selected from among chemistries I, II or III. In each case, the linkers were mixed with avidin and the mixture were allowed to react on ice for 15 to 45 minutes to form nucleation centers.

[0233] In a specific example, 1 μ g of dry hetero-bifunctional linker, such as maleimide-(CH₂)_n - biotin, where n=1 to more than 20, was first dissolved in 500 μ L of DMSO and mixed well with avidin (typically 2 to 5 molar equivalents) by vortexing for 5 seconds to create the Biotin/Avidin based Nucleation Center which contained the maleimides groups at the outer edge of the center itself. Fifteen to forty-five minutes before use, this nucleation reagent containing the nucleation centers was prepared by combining the following reagents in a 15 mL tube: 7.7 mL Detection Buffer with 440 μ L Biotinylation Reagent and 880 μ L solution of avidin in a stoichiometric ratio ranging from between 4:1 to 10:1.

[0234] In a parallel reaction, 22 μ L of a Kinase Reaction Mixture or a Phosphatase Reaction Mixture was prepared in a 15 mL tube and placed on ice: to 1500 μ L of Kinase Reaction Buffer was added 480 μ L of stock of Lipid Reagent, containing any phospholipids appropriate for optimum kinase activity, 240 μ L of a stock of fluorescent labeled peptide substrate, such as StarBright® Green-RFARKGSLRQKNV, 190 μ L of a kinase enzyme such as the theta isoform of Protein Kinase C, PKC- θ or a phosphatase enzyme. Separately 2500 μ L of ATP γ S Working Solution was prepared as follows:

Reagent 1: 5 mL, of concentrate 1 of ATP, ATP- γ NH₂ or ATP γ S with 495 mL of kinase reaction buffer in a 1.5 mL tube to make a stock ATP, ATP- γ NH₂ or ATP γ S stock solution, vortex and keep on ice;

Reagent 2: 100 μ L, of the ATP, ATP- γ NH₂ or ATP γ S Stock Solution of concentrate 2 was diluted with 2400 μ L of Kinase Reaction Buffer in a 15 mL tube to make the ATP γ S Working Solution, vortexed and kept on ice.

[0235] The kinase or phosphatase reaction and subsequent detection of the extent of phosphorylation was performed as follows:

Stage 1: 5 μ L of the Kinase Reaction Mixture was placed in each well of a 384-well microtiter plate. If any test compound was to be included, 1 μ L of the test compound at the desired concentration was added to each well. In parallel, standards were separately added to the wells used to establish a standard curve. To begin the kinase or phosphatase reactions, 5 μ L of ATP γ S substrate solution were dispensed into each well, the plate was covered to help reduce evaporation and the plate incubated at room temperature (21 to 25°C) for 45 minutes.

Stage 2: dispense 20 μ L of the Nucleation Center Reagent into each well of the microwell plate and incubated the reaction mix a second time for 2 hr at room temperature (21 to 25°C). At the end of the incubation period, read the assay plate in a fluorescence polarization microplate reader.

Example 7: Heterogeneous Fluorogenic Assay of Specific Enzyme Activities in Microtiter Plates

[0236] The theta isozyme of Protein Kinase C is used here to represent all kinase and phosphatase enzymes and the heterogeneous measurements of their activities. The only difference between the assays for the two enzyme activities is that in the former ATP or an ATP analog is used as the phosphoryl donor whereas in the case of the phosphatases, the target is also the donor. In all cases, cell lysates are added to the wells of the microtiter plate and the isozymes, if present, are allowed to act on the target that is covalently attached to the thermoplastic of Chromagen's proprietary plastic plates.

Attachment of the target to the thermoplastic

[0237] In the case of the PKC isozymes, the pseudosubstrate of PKC-alpha is a general polypeptide target for all of the PKC isozymes. By contrast, moesin, one of the ezrin polypeptides, appears to be a 73 kDa specific target of PKC-theta. Moesin was cloned and expressed as follows: cDNA to human moesin was first isolated and prepared in plasmid form, after which double stranded cDNA was generated using the polymerase chain reaction (PCR) to make two fragments of moesin using the primer pairs: 5'-CATGCCCAAACGATCAGTGT plus 5'-CAAGGCCTCCTTGGCCTCT and 5'-TTACATAGACTCAAATTCGTCAAT plus 5'-AGAGGCCAAGGAGGCCTTG. The products were cut with the appropriate restriction enzymes and either ligated or used separately. After agarose gel electrophoresis, full or partial length moesin or moesin cDNA

fragments were obtained. The primers were synthesized to allow insertion into known restrictions sites for bacterial expression vectors and the resulting DNA ligated into vectors and used for the transformation of E. coli containing pLys for expression of polypeptide. The sequence codes for a His6 tag, enabling the product to be purified using standard Ni+@ chelation resins.

[0238] Alternatively, the amino acid sequence containing the phosphorylation site KYKT*LRQIR, where the * indicates the phosphorylation site, was prepared by one of two methods: (i) as the corresponding His6 tagged peptide target, using conventional Merrifield synthesis, or (ii) as the purified CNBr fragment containing the singular KYKT*LRQIR phosphorylation target. In all cases, the His tagged recombinant protein and the terminal amino group of the His tag were used for covalent attachment to the solid phase used here. In all cases, the terminal amine groups are readily coupled to the treated polycarbonate surfaces of Chromagen's proprietary microtiter plates that are used in our HPSA gene expression assays. In a typical procedure, the wells of a plate are filled with a solution of 100 micromolar peptide target in 100 mMolar Methimidazole buffer to which were added 1 microLiter per well of Carbodiimide. The plates were incubated at 50°C overnight, after which they were washed 3 x with the appropriate kinase buffer, followed by 3x wash with distilled water after which they were vacuum dried in a lyophilization oven at 37°C.

Preparation of treated cell lysates

[0239] Phorbol 12,13-dibutyrate (PDBu) treatment of intact cells is well known as an inducer of phosphorylation of intracellular proteins by the PKC isoforms; in this case phosphorylation of the MARCKs protein target in intact cells was used as an indicator of PKC activation. Significant reduction of PDBu -induced MARCKS phosphorylation can be demonstrated in intact cells by using specific PKC isozyme inhibitors. For example, at concentrations which do not exert any negative effects on cell growth (20 nM to 6 microMolar) the PKC selective inhibitor GF109203X, a bisindolylmaleimide, that inhibits PKC isozymes by competing with enzyme - bound ATP, evokes a dose-dependent inhibition of PDBu induced MARCKS phosphorylation, demonstrating that MARCKS phosphorylation in intact cells depends in part upon the enzymatic activity of PKC. This was shown to be specifically related to inhibition of the three PKC isozymes, alpha, epsilon and theta.

[0240] Cells to be studied for kinase activation or inhibition using the assays of the present invention were first stimulated with PDBu employing standard protocols and concentrations: the stimulation of COS-1 cells is used here as a general illustration. COS-1 cells (1x10⁷/ 500 mL flask) were stably transfected with PKC theta cDNA and shown to

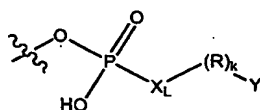
express His6 tagged PKC theta by gel analysis and PKC theta specific antibody labeling. After a 24 hour starvation period, the cells were treated for 5 minutes with varying concentrations of PDBu and or known PKC inhibitors after which the cells were lysed. The lysates were transferred immediately into the wells of the microtiter plates of the present invention which had been coated with the moesin target as described above and were then incubated in the presence of ATP or an ATP analog.

Heterogeneous measurement of the activity of PKC

[0241] Following a pre-assay-incubation of ½ hour, the plates were washed 3 times and preformed enzyme linked nucleation centers with an appropriate chemistry I, II, or III added in an approximate Mole Ratio of 2: 1 relative to the highest expected amount of phosphoryl groups. In the case of both the Nucleation centers of Type 10 A, in which the center comprises hetero-bifunctional linkers that are covalently attached to an enzyme, and the nucleation centers of Type 10 B, in which the nucleation center includes a core that is polybiotinylated, following a second incubation for between ½ hour and 60 minutes, the plates were washed again three times and the appropriate reagents added. In the case of Type 10 A nucleation centers, the subsequent addition was of 10 micromolar StarBright® Green fluorogenic substrate, the plate was simply incubated at 37°C for an additional hour and the fluorescence measured. In the case of the Type 10 B nucleation centers, the subsequent addition was of streptavidin- enzyme conjugate in a 1: 0.5 molar ratio to total biotin, after which the plate was incubated for ½ hour to 60 minutes, washed 3 times and the fluorogenic substrate added and treated as described in the case of the Type 10A nucleation centers.

WHAT IS CLAIMED IS:

1. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising a group having the formula



wherein:

X_L is selected from the group consisting of NH, NR_1 and O, wherein R_1 is selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl or heterocycloalkyl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, - NR_2 CO-, - $COOR_2$ -, -CONH-, and -CONHR₂- groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

2. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, -CO-, -NHCO-, -O-, -S-, - NR_2 CO-, - $COOR_2$ -, -CONH-, and -CONHR₂- group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group.

3. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein each R group is a bifunctional substituent independently

selected from the group consisting of unsubstituted heteroalkyl, alkyl, cycloalkyl, heterocycloalkyl, -CONH-, -NHCO- and -CO- groups.

4. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein X_L is NH.

5. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 the X_L is O.

6. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein X_L is NR_1 , wherein R_1 is selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, or heterocycloalkyl group.

7. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein k is a whole number from 1-10.

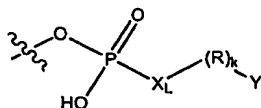
8. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein Y is selected from the group consisting of biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein and beta-lactamase.

9. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 8 wherein Y is Biotin/Avidin.

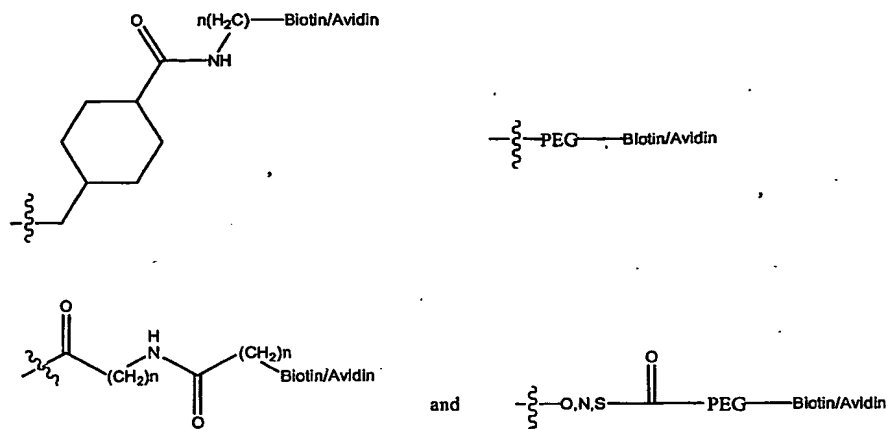
10. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein X is NH, Y is Biotin/Avidin, and each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted heteroalkyl, alkyl, cycloalkyl, heterocycloalkyl, -CONH-, -NHCO- and -CO- groups.

11. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein X is NH, Y is Biotin/Avidin, each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted heteroalkyl, alkyl, cycloalkyl, heterocycloalkyl, -CONH-, -NHCO- and -CO- groups, and k is a whole number from 1-14.

12. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 wherein the $-(R)_k-Y$ moiety of the group of the formula

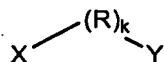


is selected from the group consisting of



Where n is a whole number from 1-25.

13. A polypeptide according to claim 1.
14. A polypeptide according to claim 9.
15. A polypeptide according to claim 11
16. An oligonucleotide according to claim 1.
17. A lipid according to claim 1.
18. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 1 further comprising a label.
19. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 11 further comprising a label.
20. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 18 wherein the label is a fluorophore.
21. A polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide according to claim 20 wherein the fluorophore is StarBright®.
22. A method of covalently attaching one or more hetero-bifunctional linkers having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl,

heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group;

to one or more hydroxyl moiety of a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising the steps of:

(a) converting the hydroxyl moiety to phosphoric acid or amino derivative of phosphoric acid; and

(b) converting the phosphoric acid or amino derivative of phosphoric acid to a phosphoro analog by reacting the phosphoric acid or amino derivative of phosphoric acid with the hetero-bifunctional linker.

23. A method according to claim 22 wherein the phosphoryl analog is a phosphoramidate, phosphoroamino or phosphorothiol analog.

24. A method according to claim 22 wherein the hydroxyl moiety is on a polypeptide containing one or more free amine or carboxylic acid group wherein the free amine or carboxylic acid group is protected before step (a).

25. A method according to claim 22 wherein the phosphoryl analog contains an amino, carboxyl or sulfhydryl group covalently attached to a detectable label.

26. A method according to claim 25 wherein the detectable label is a fluorophore.

27. A method according to claim 25 wherein the detectable label is covalently attached by reacting the label with either a homo-bifunctional linker or a hetero-bifunctional linker.

28. A method according to claim 22 wherein the hydroxyl moiety is a 5' hydroxyl group on a oligonucleotide, nucleoside or nucleotide.

29. A method according to claim 28 wherein ATP or $[\gamma\text{-NH}_2]\text{-ATP}$ is used in step (a) to convert the hydroxyl moiety to the phosphoric acid or amino derivative of phosphoric acid.

30. A method according to claim 22 wherein the hydroxyl moiety is present on a tyrosine, serine, threonine or histidine group.

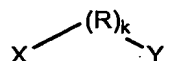
31. A method according to claim 30 wherein ATP or $[\gamma\text{-NH}_2]\text{-ATP}$ is used in step (a) to convert the hydroxyl moiety to the phosphoric acid or amino derivative of phosphoric acid.

32. A method according to claim 22 wherein the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprises a detectable label.

33. A method according to claim 32 wherein the detectable label is a fluorophore.

34. A method according to claim 22 wherein the phosphoric acid derivative is converted into a more reactive intermediate before it is reacted with the hetero-bifunctional linker to form the phosphoro analog.

35. A method of covalently attaching one or more hetero-bifunctional linkers having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO- , -NHCO- , -O- , -S- , $\text{-NR}_2\text{CO-}$, $\text{-COOR}_2\text{-}$, -CONH- , and $\text{-CONHR}_2\text{-}$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold,

polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group;

to a hydroxyl moiety of a polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprising the steps of:

- (a) converting the hydroxyl moiety to a phosphoric acid derivative; and
- (b) converting the phosphoric acid derivative to a phosphoro analog by

reacting the phosphoric acid derivative with a preformed nucleation reagent comprising a hetero-bifunctional linker.

36. A method according to claim 35 wherein the phosphoryl analog is a phosphoramidate, phosphoroamino or phosphorothioate analog.

37. A method according to claim 35 wherein the hydroxyl moiety is on a polypeptide containing one or more free amine or carboxylic acid groups wherein the free amine or carboxylic acid groups are protected before step (a).

38. A method according to claim 35 wherein the phosphoryl analog contains an amino, carboxyl or sulfhydryl group covalently attached to a detectable label.

39. A method according to claim 35 wherein the detectable label is a fluorophore.

40. A method according to claim 35 wherein the detectable label is covalently attached by reacting the label with either a homo-bifunctional linker or a hetero-bifunctional linker.

41. A method according to claim 35 wherein the hydroxyl moiety is a 5' hydroxyl group on a oligonucleotide, nucleoside or nucleotide.

42. A method according to claim 41 wherein ATP, [γ -S]-ATP or [γ -NH₂]-ATP is used in step (a) to convert the hydroxyl moiety to the phosphoric acid derivative.

43. A method according to claim 35 wherein the hydroxyl moiety is present on a tyrosine, serine, threonine or histamine group.

44. A method according to claim 43 wherein ATP, [γ -S]-ATP or [γ -NH₂]-ATP is used in step (a) to convert the hydroxyl moiety to the phosphoric acid derivative.

45. A method according to claim 35 wherein the preformed nucleation reagent comprises one or more high molecular weight reagent conjugated to one or more hetero-bifunctional linker.

46. A method according to claim 45 wherein the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, and mixed co-polymer derivatives.

47. A method according to claim 45 wherein the high molecular weight reagent is selected from polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines, and polysulfones.

48. A method according to claim 45 wherein the heterobifunctional linker is succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-6-amidocaproate or succinimidyl-3-(bromoacetamido)propionate.

49. A method according to claim 45 wherein Y of the hetero-bifunctional linker is biotin and the high molecular weight reagent is avidin.

50. A method according to claim 35 wherein the preformed nucleation reagent comprises a fluorophore.

51. A method according to claim 35 wherein the preformed nucleation reagent comprises a reactive amine group and a biotin/avidin complex.

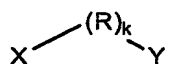
52. A method according to claim 35 wherein the polypeptide, lipid, amino acid, nucleoside, nucleotide or oligonucleotide comprises a label.

53. A method according to claim 53 wherein the label is a fluorophore.

54. A method according to claim 35 wherein the phosphoric acid derivative is converted into a more reactive intermediate before it is reacted with the nucleation reagent to form the phosphoro analog.

55. An assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a kinase substrate having one or more hydroxyl moiety;
- (b) a kinase enzyme and ATP or [γ -NH₂]-ATP; and
- (c) a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO₂, SO, and NHR₂ group; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

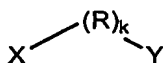
each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

56. An assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a phosphatase substrate having one or more hydroxyl moiety;
- (b) a phosphatase enzyme; and
- (c) a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

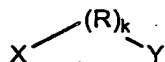
each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

57. A homogeneous assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a polypeptide substrate having one or more hydroxyl moiety;
- (b) a protein kinase enzyme and ATP or [γ -NH₂]-ATP;
- (c) a monomeric high molecular weight reagent; and
- (d) a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO₂, SO, and NHR₂ group; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

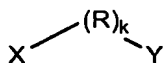
each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

58. A homogeneous assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a polypeptide substrate having one or more hydroxyl moiety;
- (b) a protein phosphatase enzyme;
- (c) a monomeric high molecular weight reagent; and
- (d) a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

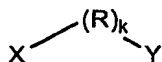
k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

59. A homogeneous assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a polypeptide substrate having one or more hydroxyl moiety;
- (b) a protein kinase enzyme, and ATP an ATP analog or any other phosphate donating group; and
- (c) a preformed nucleation reagent.

60. A homogeneous assay according to claim 59 wherein the preformed nucleation reagent is formed by attaching one or more high molecular weight reagent to one or more hetero-bifunctional linkers having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

61. A homogeneous assay according to claim 60 wherein the hetero-bifunctional linker has a reactive amino terminus.

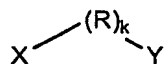
62. A homogeneous assay according to claim 60 wherein the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines and polysulfones.

63. A homogeneous assay according to claim 60 wherein the high molecular weight reagent is avidin and the hetero-bifunctional linker comprises biotin.

64. A homogeneous assay for detecting phosphorylation or de-phosphorylation comprising:

- (a) a polypeptide substrate having one or more hydroxyl moiety;
- (b) a protein phosphatase enzyme; and
- (c) a preformed nucleation reagent.

65. A homogeneous assay according to claim 64 wherein the preformed nucleation reagent is formed by attaching one or more high molecular weight reagents with one or more hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO₂, SO, and NHR₂ group; wherein R₂ is selected

from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

66. A homogeneous assay according to claim 65 wherein the X group of the hetero-bifunctional linker is an amine.

67. A homogeneous assay according to claim 66 wherein the high molecular weight reagent is avidin and the hetero-bifunctional reagent further comprises biotin.

68. A homogeneous assay according to claim 65 wherein the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines, and polysulfones.

69. A assay for detecting phosphorylation or de-phosphorylation comprising the steps of:

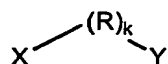
- (a) attaching a fluorophore to a polypeptide;
- (b) blocking at least one reactive free amine or acid group on the polypeptide to form a protected polypeptide;
- (c) phosphorylating the protected polypeptide with ATP, an ATP analog or any other phosphate donating substrate; and
- (d) measuring fluorescence polarization.

70. A method for blocking one or more reactive amine or acid groups on a polypeptide target comprising:

(a) treating the polypeptide target with a fluorescein-NHS or StarBright® Dye Isothiocyanate; and then

(b) protecting one or more free amines or acids present on the polypeptide target.

71. A method of preparing a preformed nucleation center comprising the step of reacting one or more high molecular weight reagent with one or more hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO₂, SO, and NHR₂ group; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO-, -NHCO-, -O-, -S-, -NR₂CO-, -COOR₂-, -CONH-, and -CONHR₂- groups; wherein R₂ is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

72. A method according to claim 71 wherein the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines and polysulfones.

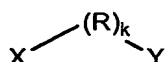
73. A method according to claim 71 wherein the hetero-bifunctional linker has a reactive amino group.

74. A method according to claim 71 wherein the high molecular weight reagent is avidin and the hetero-bifunctional linker comprises biotin.

75. A method according to claim 74 wherein the hetero-bifunctional linker further comprises a reactive amino group.

76. An assay for the detection of phosphorylation and de-phosphorylation comprising:

- (a) combining a protein kinase, one or more test compounds, a labeled polypeptide substrate, and ATP or $[\gamma\text{-NH}_2]\text{-ATP}$ an ATP to make a first mixture;
- (b) allowing the first mixture to react to form a phosphopeptide product;
- (c) adding a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, -CO- , -NHCO- , -O- , -S- , $\text{-NR}_2\text{CO-}$, $\text{-COOR}_2\text{-}$, -CONH- , and $\text{-CONHR}_2\text{-}$ group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group;

to create a phosphorylated polypeptide product; and

- (d) measuring the fluorescence polarization of the phosphorylated polypeptide product.

77. An assay according to claim 76 wherein the Y group of the hetero-bifunctional linker is biotin.

78. An assay according to claim 76 wherein avidin is added to the phosphorylated polypeptide product before step (d).

79. An assay according to claim 76 wherein the hetero-bifunctional linker is present in a preformed nucleation mixture comprising the hetero-bifunctional linker and a high molecular weight reagent.

80. An assay according to claim 68 wherein the hetero-bifunctional linker covalently binds to an amine group of the phosphopeptide product to form the phosphorylated polypeptide product.

81. An assay according to claim 76 wherein the labeled polypeptide substrate is labeled with a fluorophore.

82. An assay according to claim 76 wherein the phosphopeptide product is formed in less than three hours.

83. An assay according to claim 82 wherein the phosphopeptide product is formed in less than one hours.

84. An assay according to claim 83 wherein the phosphopeptide product is formed in less than 30 minutes.

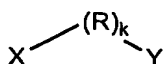
85. An kit comprising the materials for the assay of claim 76.

86. A kit according to claim 85 in a configuration selected from microtiter plates, fluorescence microwell plate readers, microfluidic devices, bead-based separatory devices, fluorescence microscopes and electrophoresis gels.

87. An assay for the detection of phosphorylation and de-phosphorylation comprising:

- (a) combining a protein kinase, one or more test compounds, a labeled polypeptide substrate, and ATP or an ATP analog to make a first mixture;
- (b) allowing the first mixture to react to form a phosphopeptide product;
- (c) adding a preformed nucleation reagent to create a phosphorylated polypeptide product; and
- (d) measuring the fluorescence polarization of the phosphorylated polypeptide product.

88. An assay according to claim 87 wherein the preformed nucleation reagent comprises a high molecular weight reagent associated with a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

89. An assay according to claim 88 wherein Y of the hetero-bifunctional linker is biotin and the high molecular weight reagent is avidin.

90. An assay according to claim 88 wherein the hetero-bifunctional linker covalently binds to an amine or thio group of the phosphopeptide product to form the phosphorylated polypeptide product.

91. An assay according to claim 87 wherein the ATP analog is $[\gamma\text{-S}]\text{-ATP}$ or $[\gamma\text{-NH}_2]\text{-ATP}$.

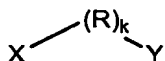
92. An assay according to claim 87 wherein the labeled polypeptide substrate is labeled with a fluorophore.

93. An assay according to claim 87 wherein the phosphopeptide product is formed in less than three hours.

94. An assay according to claim 93 wherein the phosphopeptide product is formed in less than one hour.

95. An assay according to claim 94 wherein the phosphopeptide product is formed in less than 30 minutes.

96. An kit comprising the materials for the assay of claim 87.
97. A kit according to claim 96 in a configuration selected from microtiter plates, fluorescence microwell plate readers, microfluidic devices, bead-based separatory devices, fluorescence microscopes and electrophoresis gels.
98. A kit for the detection of phosphorylation or de-phosphorylation comprising:
- (a) a target substrate;
 - (b) a reaction buffer;
 - (c) an enzyme;
 - (d) ATP or an ATP analog; and
 - (e) a nucleation reagent.
99. A kit according to claim 98 wherein the target substrate is a polypeptide target.
100. A kit according to claim 99 wherein the polypeptide target is from 5-500 amino acids.
101. A kit according to claim 100 wherein the ATP analog is $[\gamma\text{-S}]\text{-ATP}$ or $[\gamma\text{-NH}_2]\text{-ATP}$.
102. A kit according to claim 98 wherein the target substrate is labeled.
103. A kit according to claim 102 wherein the target substrate is labeled with a fluorophore.
104. A kit according to claim 98 wherein the nucleation reagent comprises a high molecular weight reagent and a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavidin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter plates, silicon plates, and polymers comprising at least one active functional group.

105. A kit according to claim 104 wherein the high molecular weight reagent is avidin.

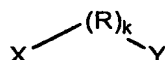
106. A kit according to claim 104 wherein the high molecular weight reagent and the hetero-bifunctional linker are stored separately.

107. A kit according to claim 98 wherein the target substrate, reaction buffer, enzyme, ATP or ATP analog, and nucleation reagent are kept separate from each other until used.

108. A kit according to claim 98 in a homogeneous assay configuration selected from microtiter plates, fluorescence microwell plate readers, microfluid devices, bead-separatory devices, and electrophoresis gels.

109. A preformed nucleation reagent comprising:

- (a) a high molecular weight reagent; associated with
- (b) a hetero-bifunctional linker having the formula



wherein:

X is selected from the group consisting of NH_2 , succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol, SO_2 , SO, and NHR_2 group; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

each R group is a bifunctional substituent independently selected from the group consisting of unsubstituted or substituted alkyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, $-\text{CO}-$, $-\text{NHCO}-$, $-\text{O}-$, $-\text{S}-$, $-\text{NR}_2\text{CO}-$, $-\text{COOR}_2-$, $-\text{CONH}-$, and $-\text{CONHR}_2-$ groups; wherein R_2 is selected from hydrogen and unsubstituted alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl and heteroaryl group;

k is a whole number from 1 to 100; and

Y is selected from from the group consisting of biotin, biotin/avidin, biotin/streptavidin, alkaline phosphatase, casein, beta-lactamase, Bovine Serum Albumin, IgG, Avidin-alkaline phosphatase, streptavin-alkaline phosphatase, and biotin or streptavidin complexed with one or more group selected from glycoproteins, enzymes, antibodies, nucleotides, polypeptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and solid surfaces selected from microtiter pates, silicon plates, and polymers comprising at least one active functional group.

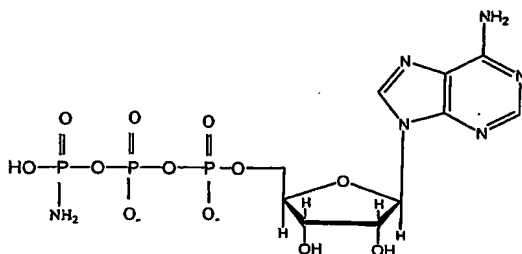
110. A preformed nucleation reagent according to claim 109 wherein the high molecular weight reagent is selected from oligomeric polymers, synthetic polymers, mixed co-polymer derivatives, polyamino acids, polypeptides, polylysine, poly acrylic acid, polyamines, and polysulfones.

111. A preformed nucleation reagent according to claim 109 wherein the X group of the hetero-bifunctional linker is an amine.

112. A preformed nucleation reagent according to claim 109 wherein the high molecular weight reagent is avidin and Y on the hetero-bifunctional linker is biotin.

113. A method according to claim 109 wherein the hetero-bifunctional linker comprises a reactive amino group.

114. A compound of the formula



General Structure of Heterobifunctional Linkers



R= Alkyl, cycloalkyl, cycloalkyl-alkyl, aromatic, alkyl-aromatic, stilbene, heterocyclic, alkyl-heterocyclic, CH₂CH₂-O-, alkyl-CH₂CH₂-O-alkyl, CH₂-CH=CH-, CH₂-NHCO, alkyl-NHCO-alkyl, CH₂CH₂-S-, CH₂CH₂-NH-, Long Chain Alkyl Amino, etc.

X = NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol,

Y = Biotin

= Biotin/Avidin
 = Biotin/Streptavidin (SA)
 = Alkaline Phosphatase (AP)
 = Casein
 = beta-Lactamase
 = BSA
 = IgG
 = Avidin-AP
 = Streptavidin-AP
 = Biotin or Streptavidin complexed with :

Glycoproteins, enzymes, antibodies, DNA, RNA, peptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and other solid surfaces such as microtitre plates, glass (silicon) plates, and any other polymer comprised of active functions, for example, -OH, -NH₂, -SH, succinimido, maleimido groups.

Figure 1. General chemical structure and compositions of the heterobifunctional linkers of the Present Invention

Figure 2. Classification of Kinases and Phosphatases by Target Structure

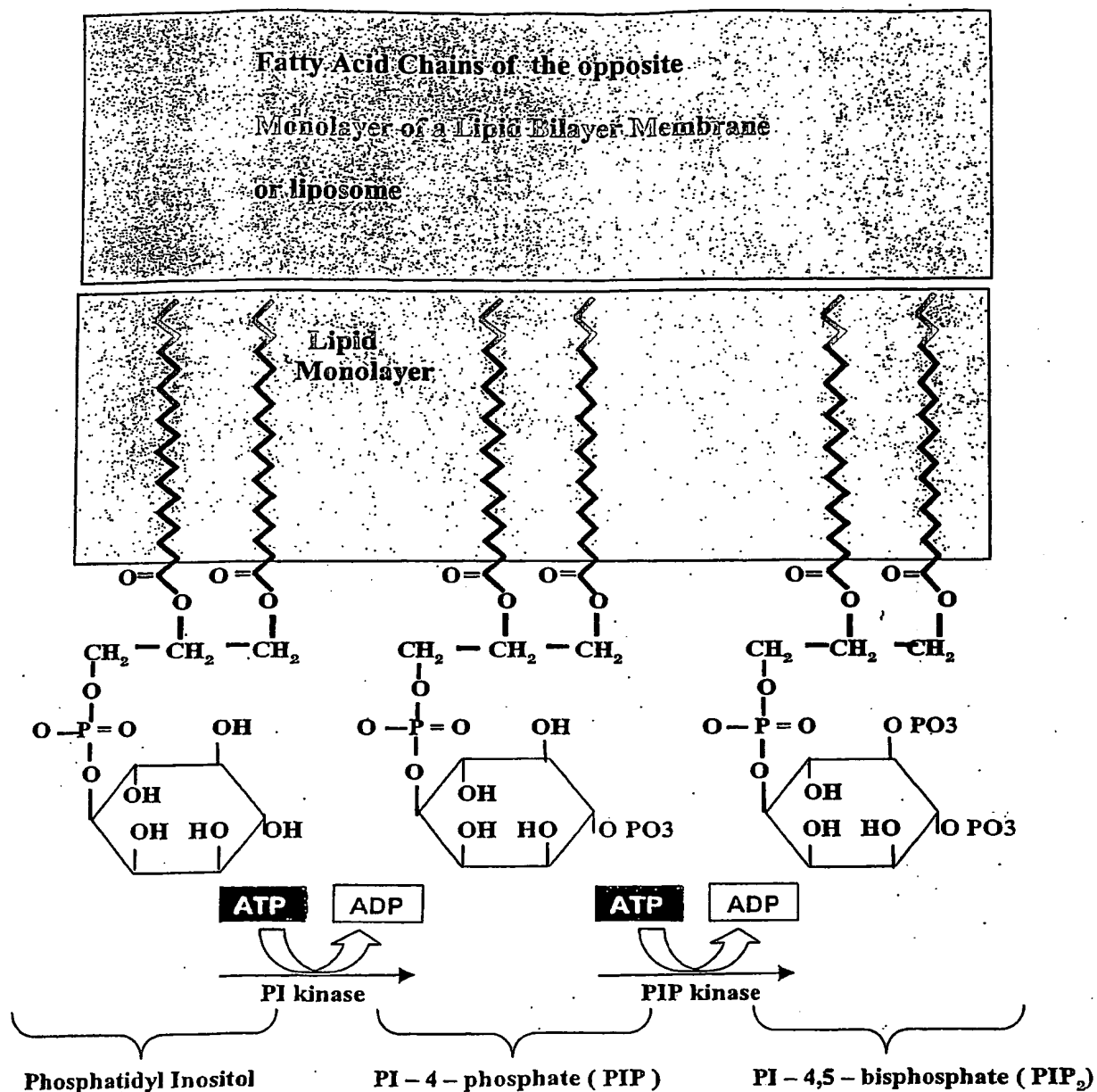
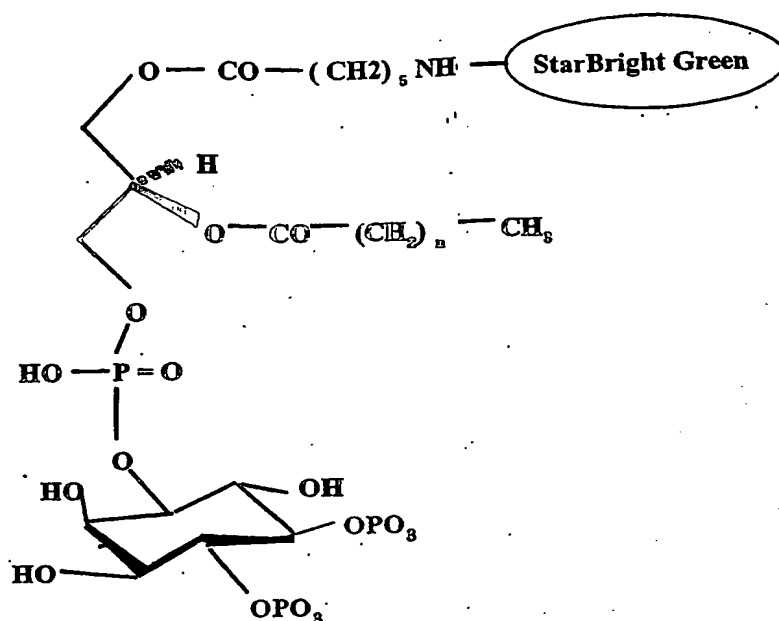


Figure 2a . a) representative water insoluble target and sites of specific actions of lipid kinases. Phosphatidyl Inositol and the Site specific actions of two lipid kinases



STARBRIGHT GREEN - PHOSPHATIDYLINOSITOL- 4,5- BISPHOSPHATE
[STARBRIGHT GREEN - PtdIns(4,5)P2]

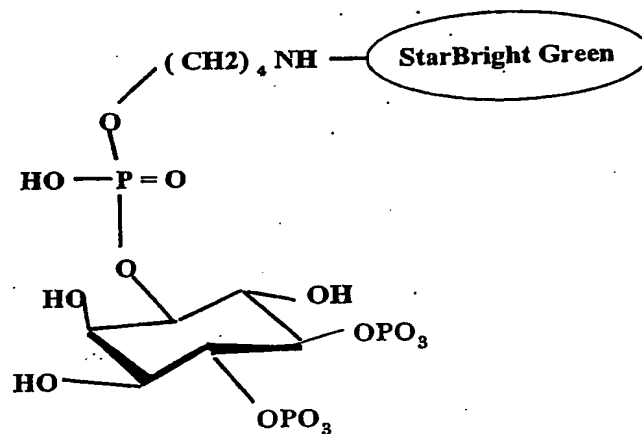


Figure 2.b. Water soluble lipid kinase target substrates: above, an example of the water soluble, StarBright-labeled derivatives of phosphatidyl inositol and its phosphorylated products. Alternative target substrates may be the single fatty acyl chain 1-StarBright Green -myo - inositol -1 phosphate lithium salts shown below and described in the text.

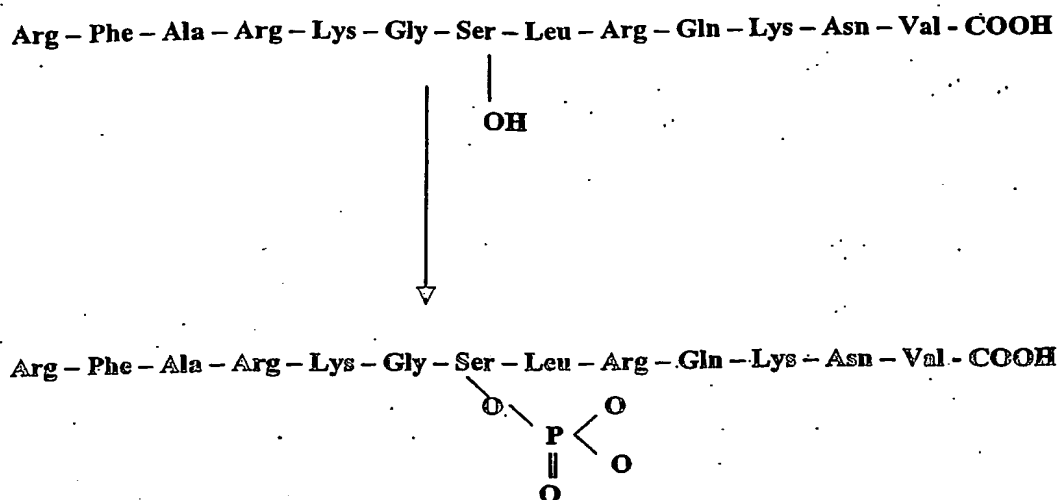


Figure 2. c. Peptide Target Substrate Phosphorylation -
 The pseudosubstrate of Protein Kinase C-alpha and the site specific
 Phosphorylation of Serine by the PKC isozyme, PKC-theta

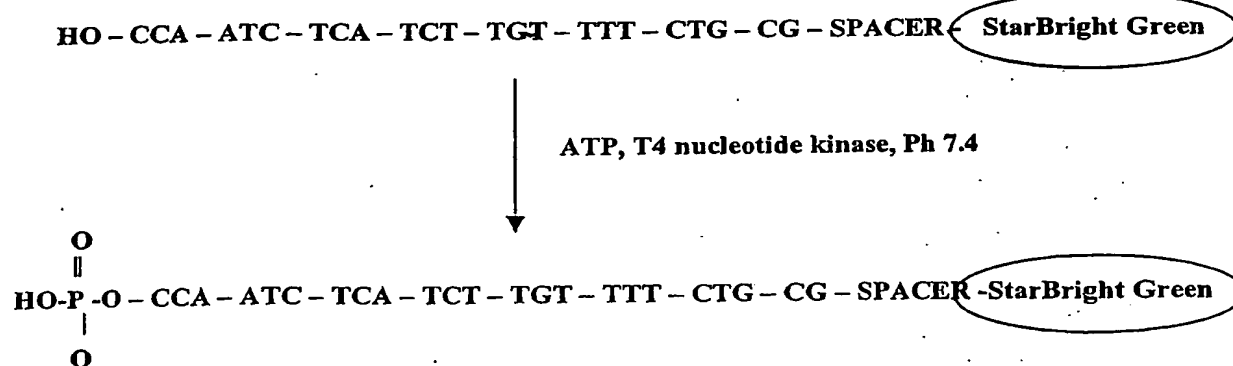
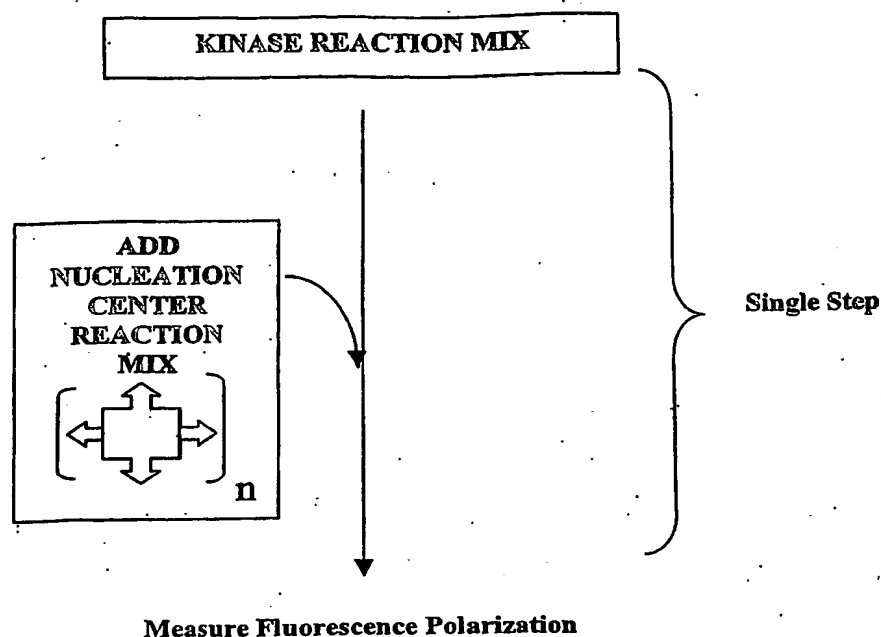
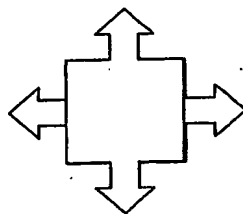


Figure 2.d. Oligonucleotide Target Substrate Phosphorylation -
 The beta-actin target of T4 nucleotide kinase and the terminal phosphorylation
 of the oligonucleotide by the kinase

a) Single Step *Homogeneous* Assay using the rapid reaction method of the Present Invention



b) the "Nucleation Effect" in which multiple heterobifunctional linkers are attached to High Molecular weight core molecules such as avidin or another polymer to create a multi-valent reaction center that serves to enhance reaction rates,



where the square at the center represents the high molecular weight core that is conjugated to multiple copies ($n > 2$) of the heterobifunctional linkers (arrow heads) shown in Figure 1.

Figure 3. Schematic diagram (a) of the single step *homogeneous* assay method based upon the "nucleation effect" of the present invention and an idealized diagram (b) illustrating the nucleation effect itself;

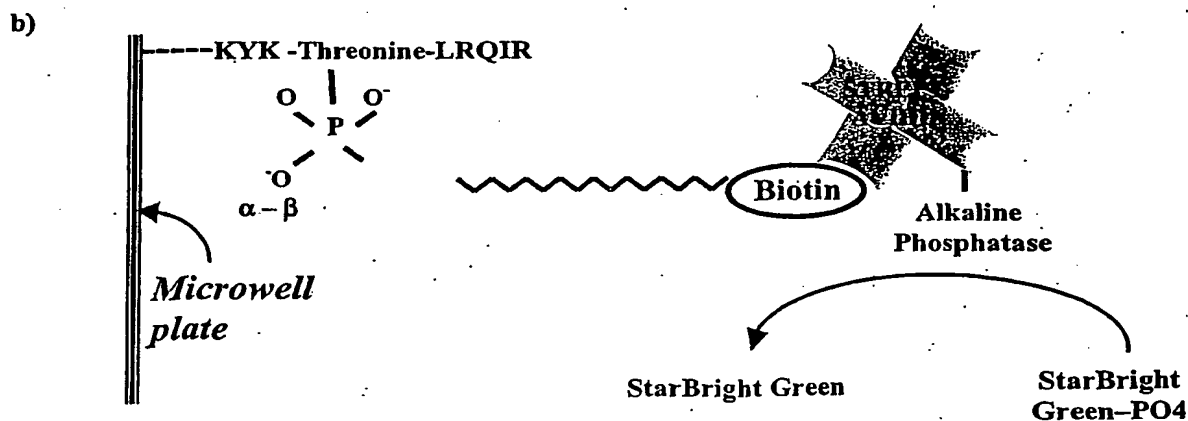
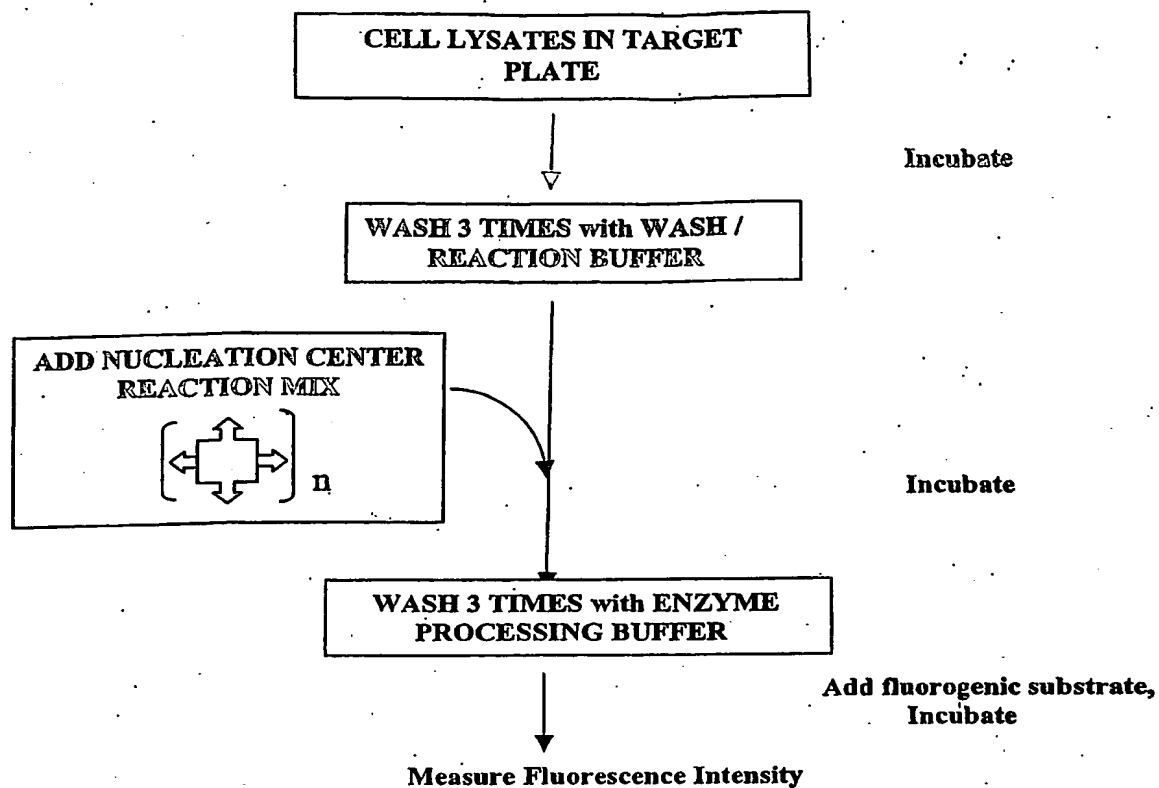
a) Multi- Step *Heterogeneous* Assay of the Present Invention

Figure 4. Schematic diagram (a) and mechanism (b) of the *heterogeneous* assay method based upon the nucleation effect of the present invention

Phosphoramidate Chemistry For Developing Fluorescence Polarization Based Protein Kinase Assays

Schematic Representation of Steps Involved:

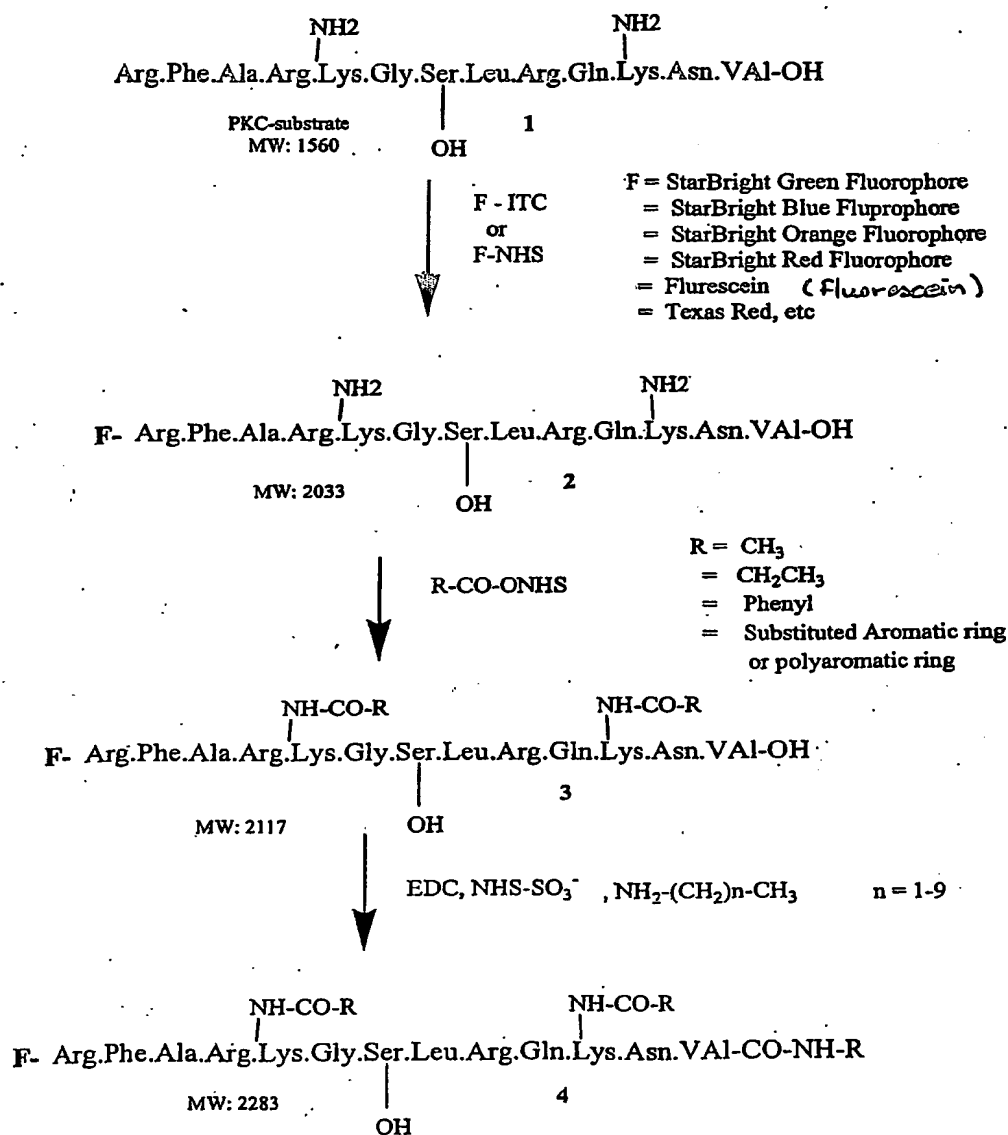


Figure 5. Novel protocols for blocking potentially reactive -NH₂ and -COOH groups on peptide targets of the present invention

Applied Biosystems Voyager System 1197

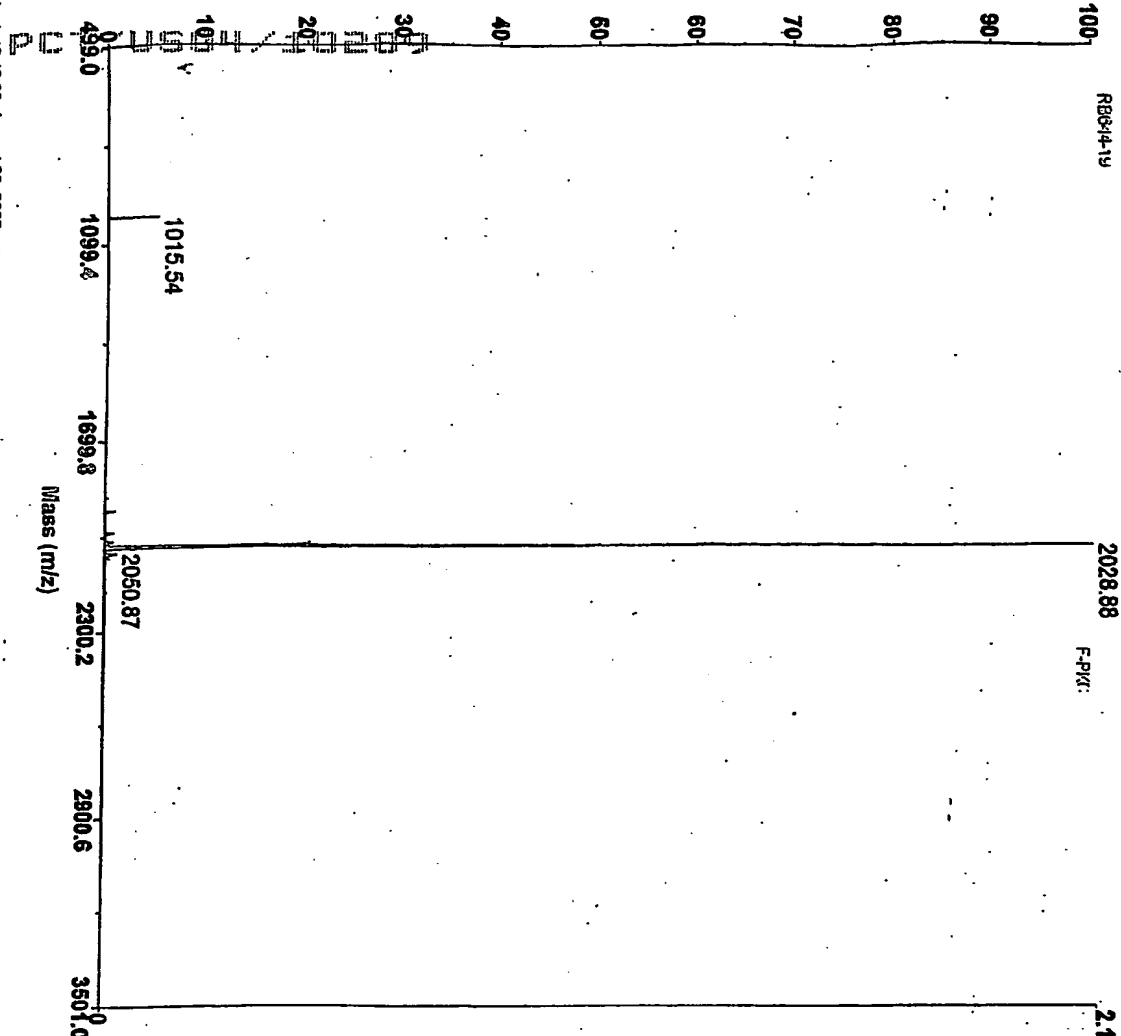
Voyager Spec #1197 = 2028.9, 20777

WO 2004/089295

PCT/US2004/010289

8/31

Intensity



2.1E+4

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire Q: 0.05%
 Extraction delay time: 200 msec

Acquisition mass range: 500 - 3500 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1546

Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: 8-Cyano-4-hydroxycinnamic acid
 Low mass gate: 500 Da

Digitizer start time: 14.268
 Bin size: 2 msec
 Number of data points: 11636
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 01
 Plate ID: 1
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.pit
 Lab name: PE Biosystems

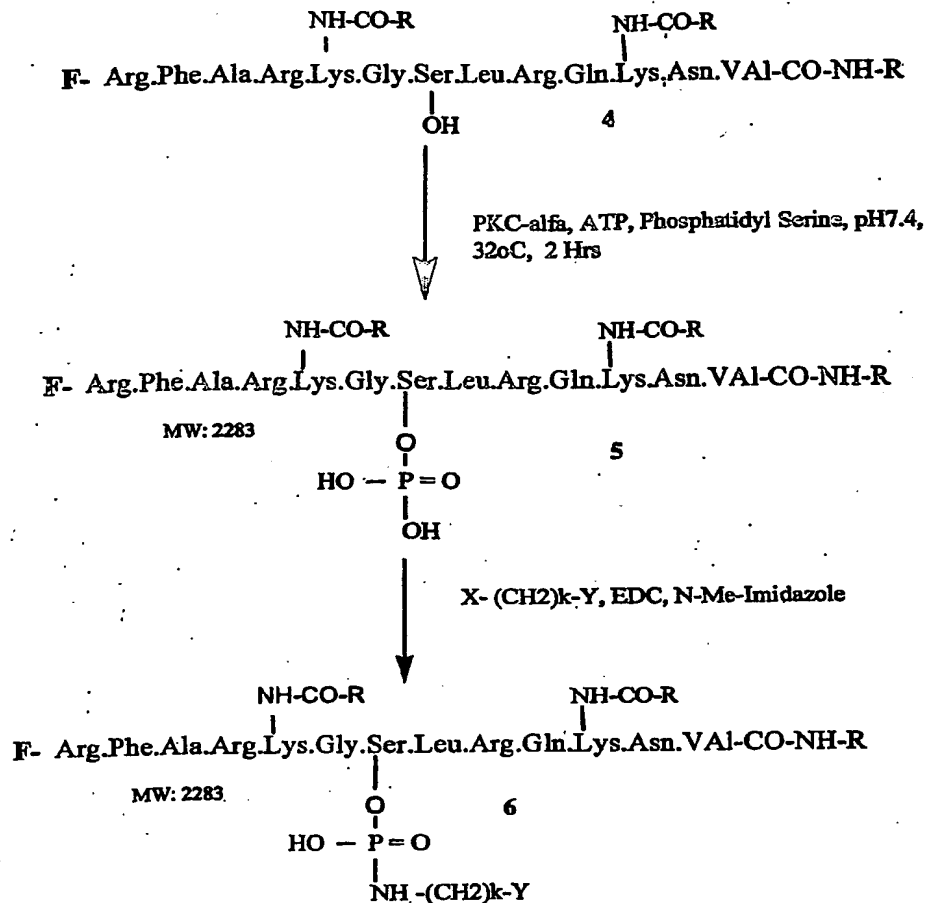
Absolute x-position: 1398.02
 Absolute y-position: 47095.3
 Relative x-position: -189.478
 Relative y-position: -212.183
 Shots in spectrum: 100
 Source pressure: 1.178e-008
 Mirror pressure: 0
 TC2 pressure: 0.0134
 TIS gate width: 30
 TIS flight length: 940

Printed: 12:43:00, August 23, 2002

AcqAgentRAMR084-18 Fm08-7 August23, 02_0001.dci

Figure 6.

Maldi-Mass spectrum of the PKC-peptide target labeled with fluorescein at its N-terminal for the kinase activities of the isoforms of Protein Kinase C (PKC)



Where $k = 1-15$

and Y = biotin, biotin-avidin complex, biotin-streptavidin complex, avidin-alkaline phosphatase (AP) conjugate, or unconjugated AP, b-Lactamase, Casein, or any other large molecular weight, including but not limited to antibodies, and derivatized particles.

Figure 7. Protocol and chemistry of the present invention for the formation of phosphor-amidates used in the detection of phosphoryl groups using the Nucleation Centers and rapid assay methods and phosphoramidate Chemistry I of the present invention

10/31

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 2199.9, 101171

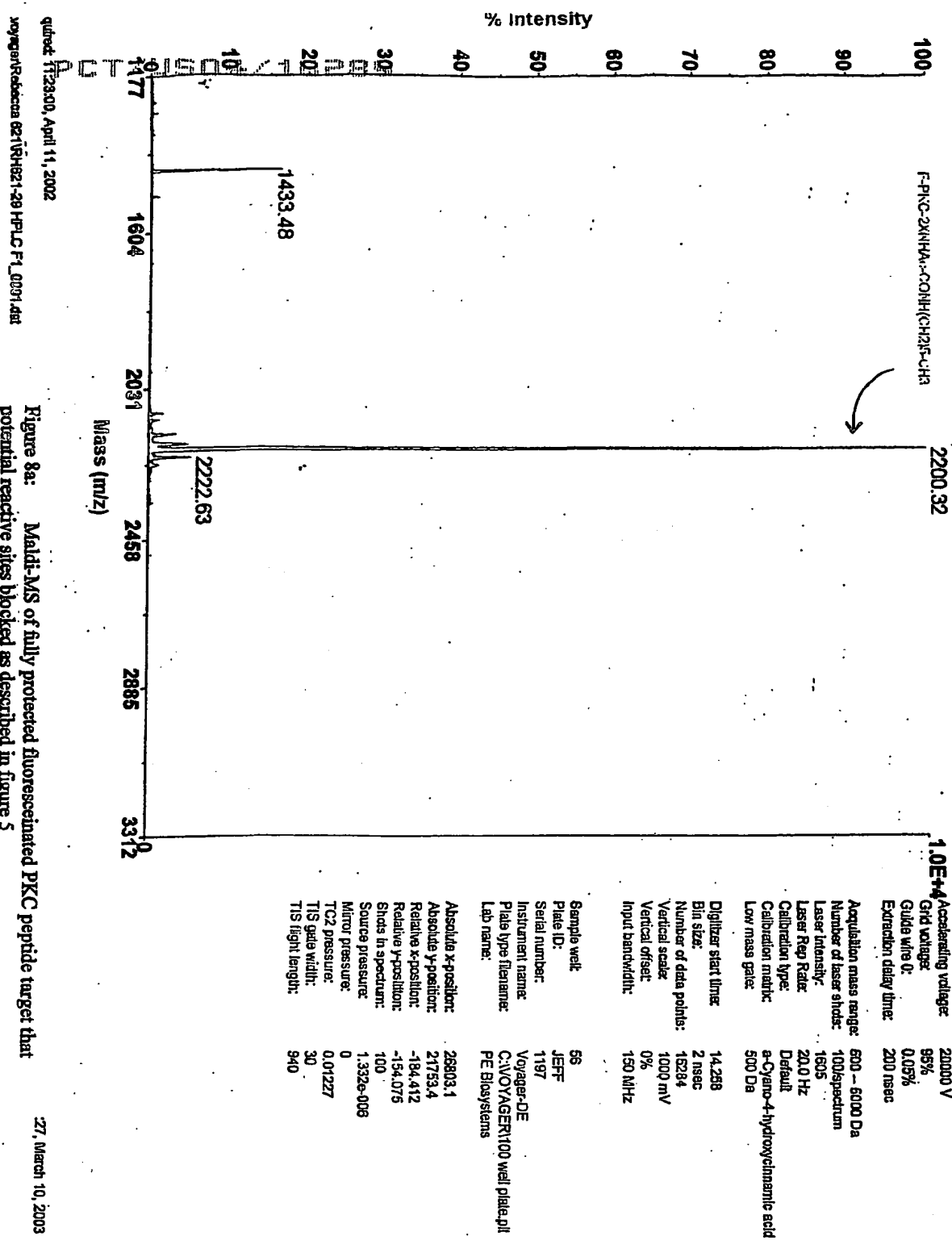


Figure 8c: MALDI-MS of fully protected fluoresceinated PKC peptide target that potential reactive sites blocked as described in figure 5

27, March 10, 2003

11/31

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 2279.6, 3814

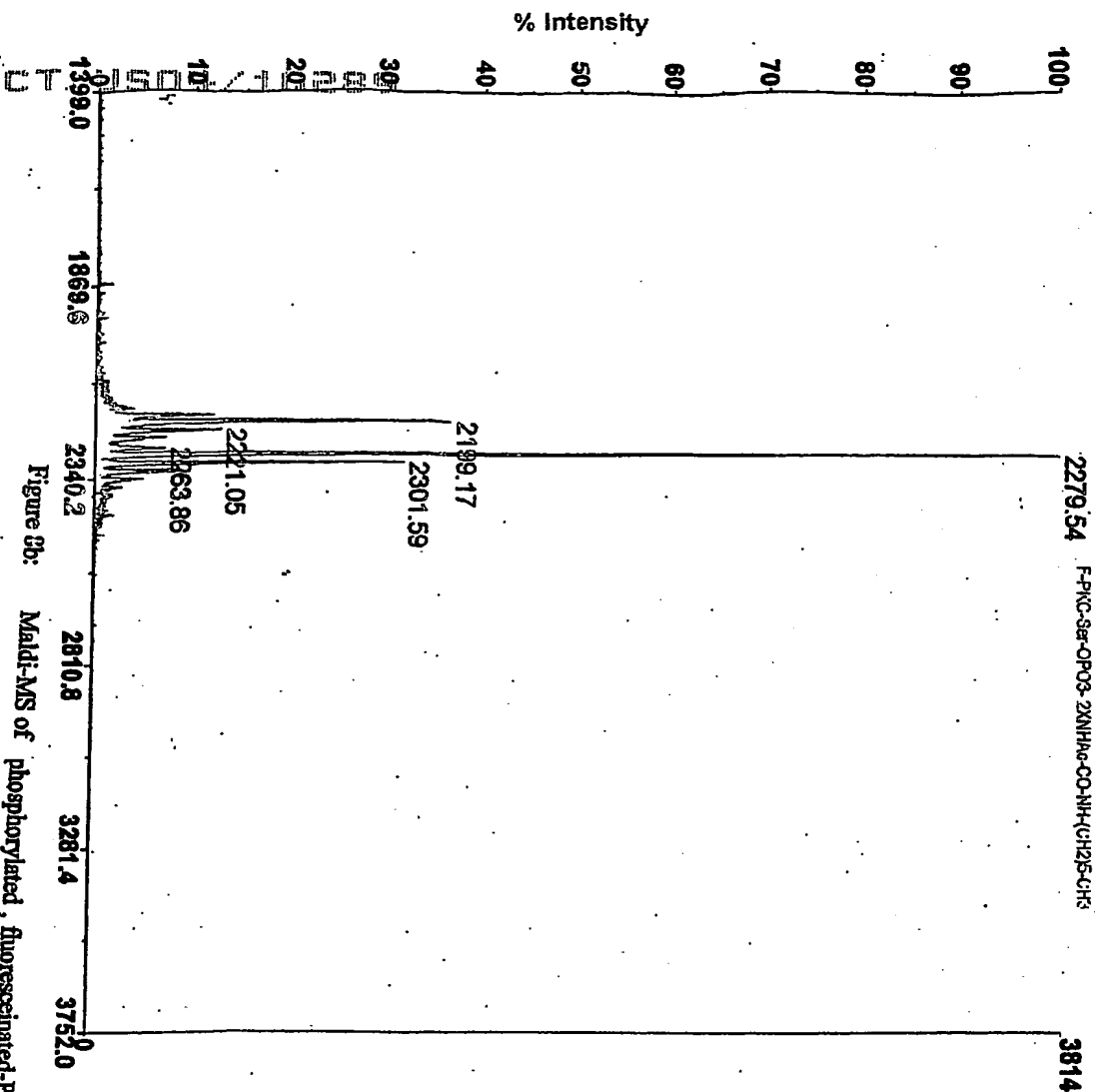


Figure 8b:

quiet: 080300, April 23, 2002
 voyagerRedocera 6218H21-40 32°C 010112.1

Maldi-MS of phosphorylated, fluoresceinated-PKC peptide target that had potential sites blocked as described in Example 1 *before* the addition of multiplexed Nucleation Centers that had been performed from avidin and the heterobifunctional biotin linkers of chemistry 1.

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

Accelerating voltage: 20000 V
 Grid voltage: 99%
 Guide wire D: 0.05%
 Extraction delay time: 200 nsec

Acquisition mass range: 600 - 6000 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1940
 Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: e-Cyano-4-hydroxycinnamic acid
 Low mass gate: 600 Da

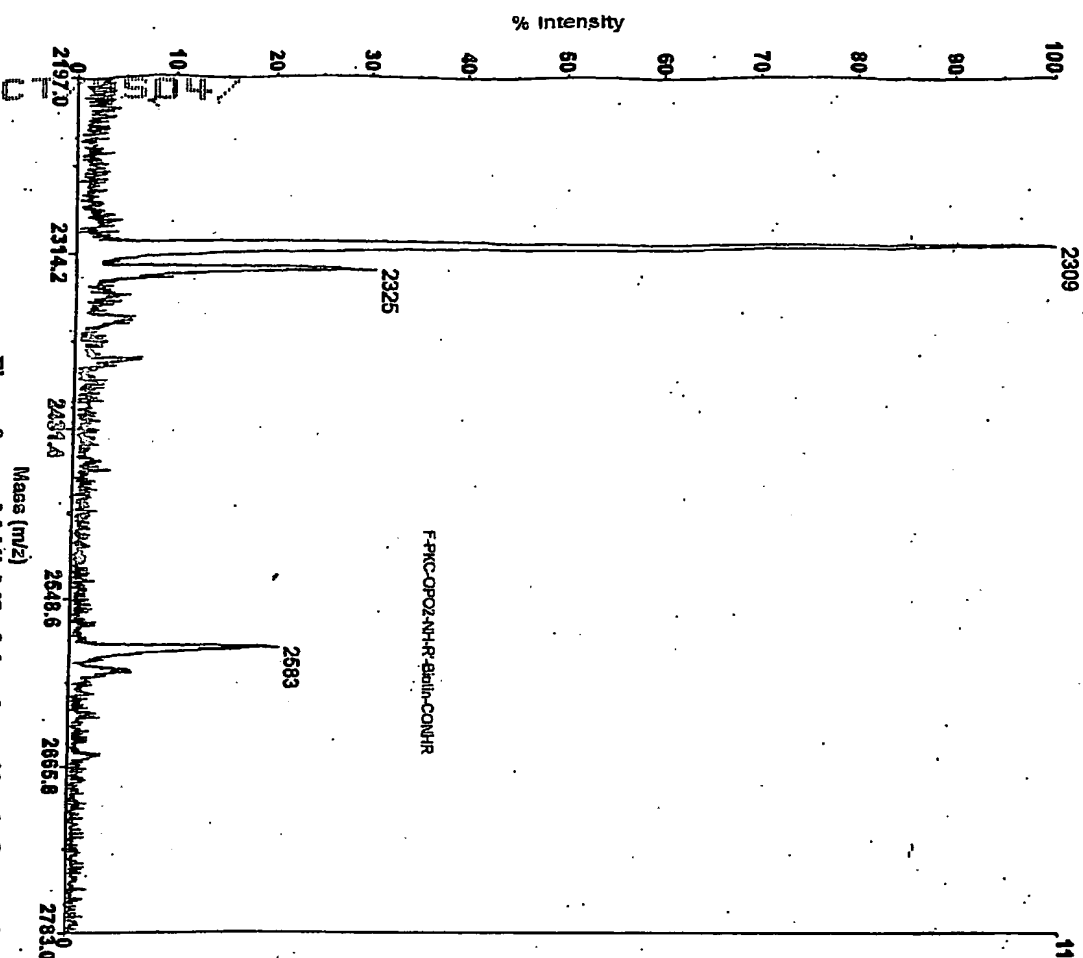
Digitizer start time: 14.258
 Bin size: 2 nsec
 Number of data points: 16284
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 55
 Plate ID: JEFF
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.plt
 Lab name: PE Biosystems

Absolute X-position: 21178.3
 Absolute Y-position: 20890.3
 Relative X-position: -728.201
 Relative Y-position: -1017.22
 Shots in spectrum: 100
 Source pressure: 6.258e-007
 Mirror pressure: 0
 TIS pressure: 0.00944
 TIS gate width: 30
 TIS flight length: 940

Applied Biosystems Voyager System 1197

Voyager Spec #1BP = 1762.0, 133041



F-PKC-OPOL-NH-R-Biotin-CON-HR

Mass (m/z)

Figure 8c:

Malik: MS of phosphoramidated, fluoresceinated PKC peptide target that had potential reactive sites blocked before the addition of multiplexed preformed Nucleation Centers from avidin and heterobifunctional biotin

Acquired: 173000, November 19, 2001
VoyagerRAMBIOm110v18_01_0001.dai

Printed: 18:03, March 10, 2003

Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

1162.0 Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire D: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 1500 - 3000 Da
Number of laser shots: 1000/spectrum
Laser intensity: 1861
Laser Rep Rate: 20.0 Hz
Calibration type: External - D:\voyager\data\level2 nov13_0001.cal
Calibration matrix: a-Cyano-4-hydroxycinnamic acid
Low mass gate: 1500 Da

Digitizer start time: 24.69
Bin size: 2 nsec
Number of data points: 5089
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 01
Plate ID: 1
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate pit
Lab name: PE Biosystems

Absolute x-position: 1883.1
Absolute y-position: 47312.8
Relative x-position: 295.604
Relative y-position: 5.30646
Stitch in spectrum: 100
Source pressure: 4.304e-007
Mirror pressure: 0
TIC2 pressure: 0.00875
TIS gate width: 30
TIS flight length: 840

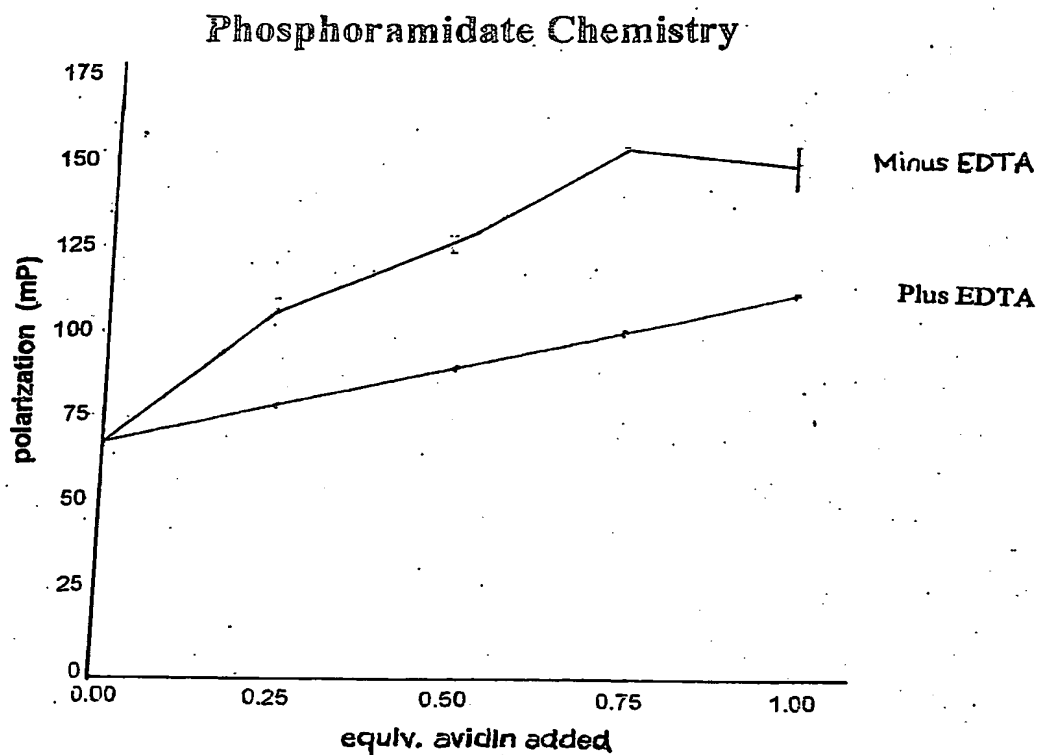
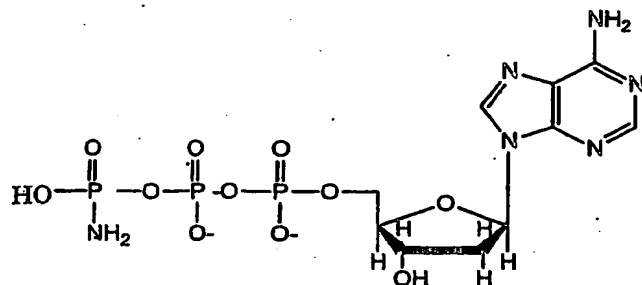


Figure 9. Fluorescence Polarization analysis of the stoichiometry of Nucleation Center Binding. The phosphoramidated PKC peptide target shown in Figure 7 after the addition of varying amounts of multiplexed Nucleation Centers using the linkers of Chemistry I. The two samples differed in that the negative controls were performed in the presence of 5mMolar EDTA which destroys the activity of the kinase.

Structure of γ -NH₂-ATP:

7

Figure 10. Chemical structure of the ATP structural analog, γ -Amino ATP (γ -NH₂-ATP)

Synthesis of γ -Amino-ATP

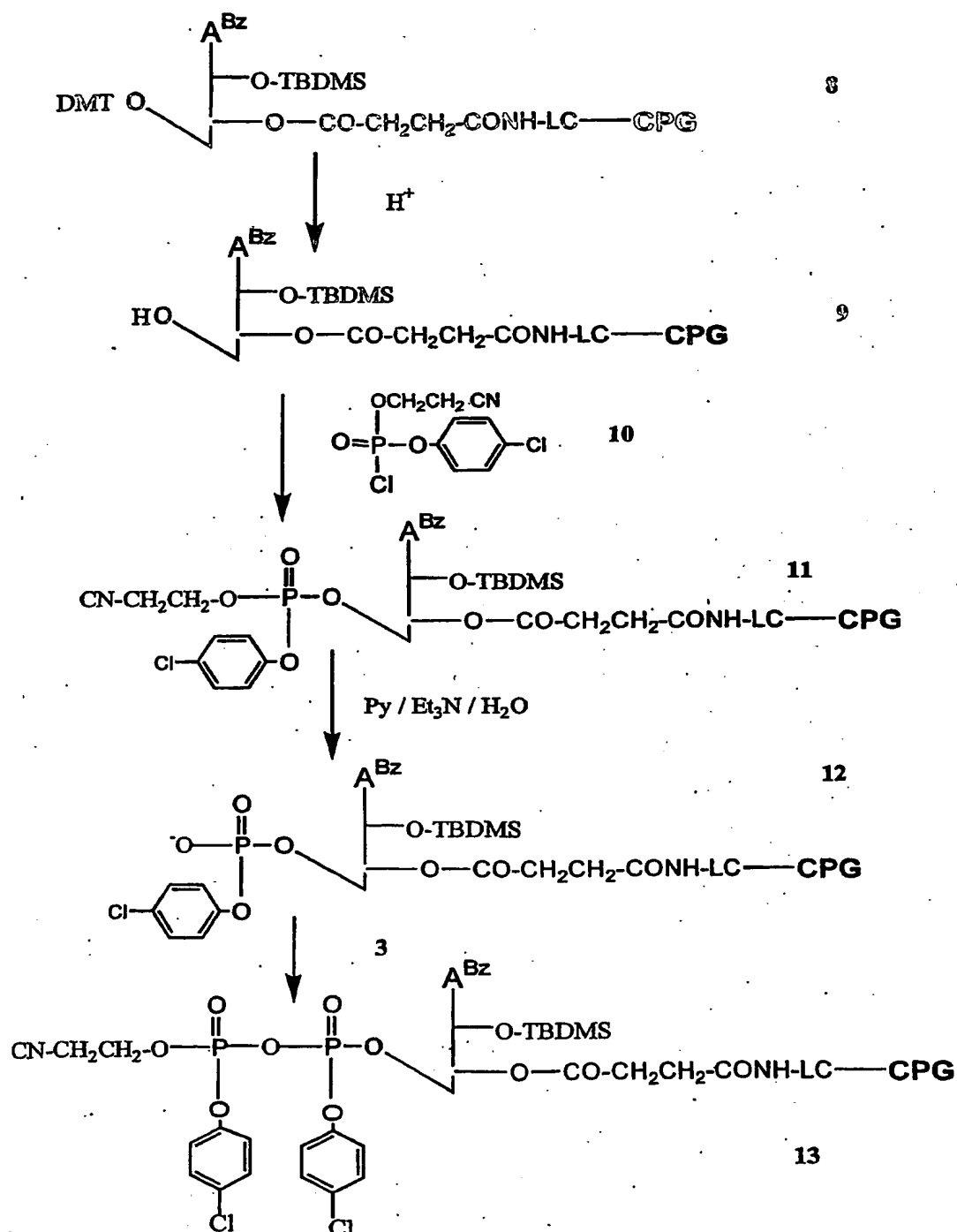


Figure 11. Protocol and chemistry of the present invention for the synthesis of γ -NH₂-ATP

" Scheme for the synthesis of gamma-Amino-ATP continues

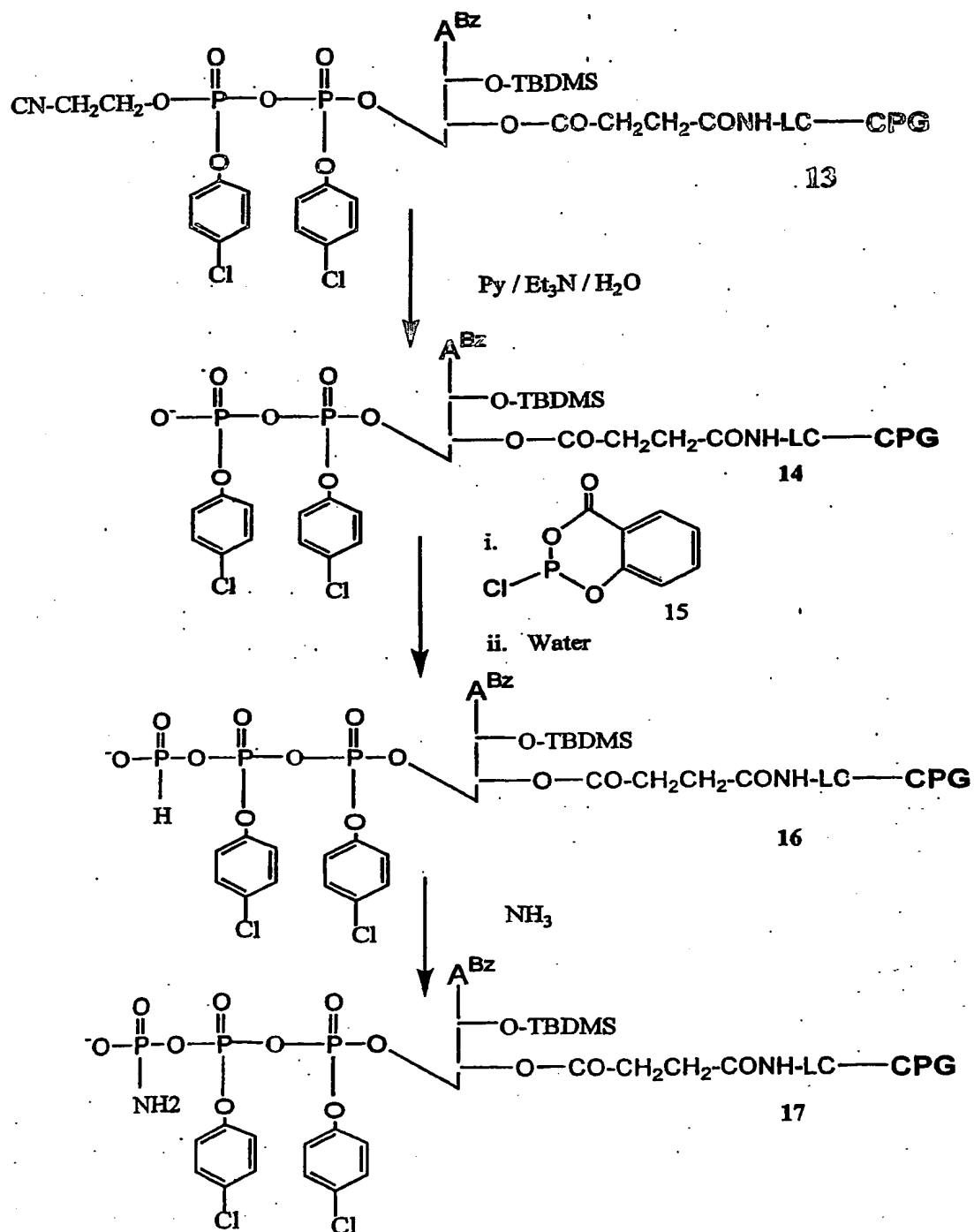
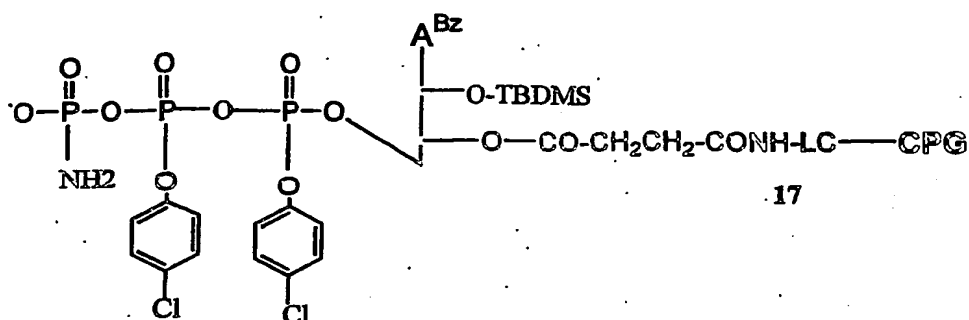


Figure 11: continuation (page 2)

Page 3 of Synthesis of γ -Amino-NH₂

- i. TMG, RT, 4 Hours
ii. NH₄OH, 60°C, 8 hours
iii. Concentrate to dryness under vacuum
iv. TBAF, RT, 16 hours

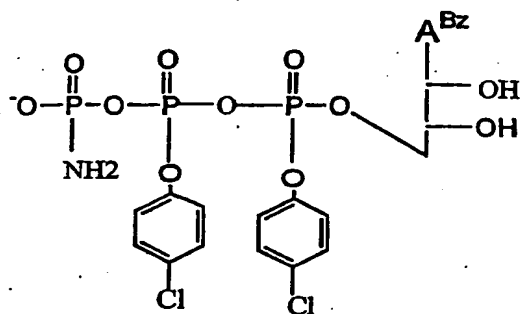
 γ -Amino-NH₂

Figure 11: continuation (page 3)

Figure 12. Procedure for phosphoroamidation of fluoresceinated -PKC peptide target using PKC- α and γ -NH₂-ATP

Phosphorothioate Chemistry

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH

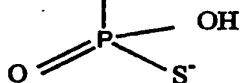
OH

2

gamma-S-ATP, PKC-alfa

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH

20



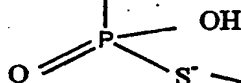
↓

X

(R)_k

Y

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH



(CH₂)_k

Y

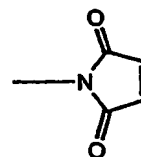
21

where k= 1-100

R= Alkyl, alkoxy, cycloalkanyl,
aromatic, heterocyclic,
ethylene glycolic, peptidyl, etc

Y= Biotin, Biotin-Avidin,
Biotin-Streptavidin, or Large
Polymer such as Alkaline
Phosphatase (AP), Streptavidin
(SA), Casein, glycoprotein, IgG,
enzyme, DNA, RNA with or
without conjugation to Avidin

X =



X = I-CH₂CONH--

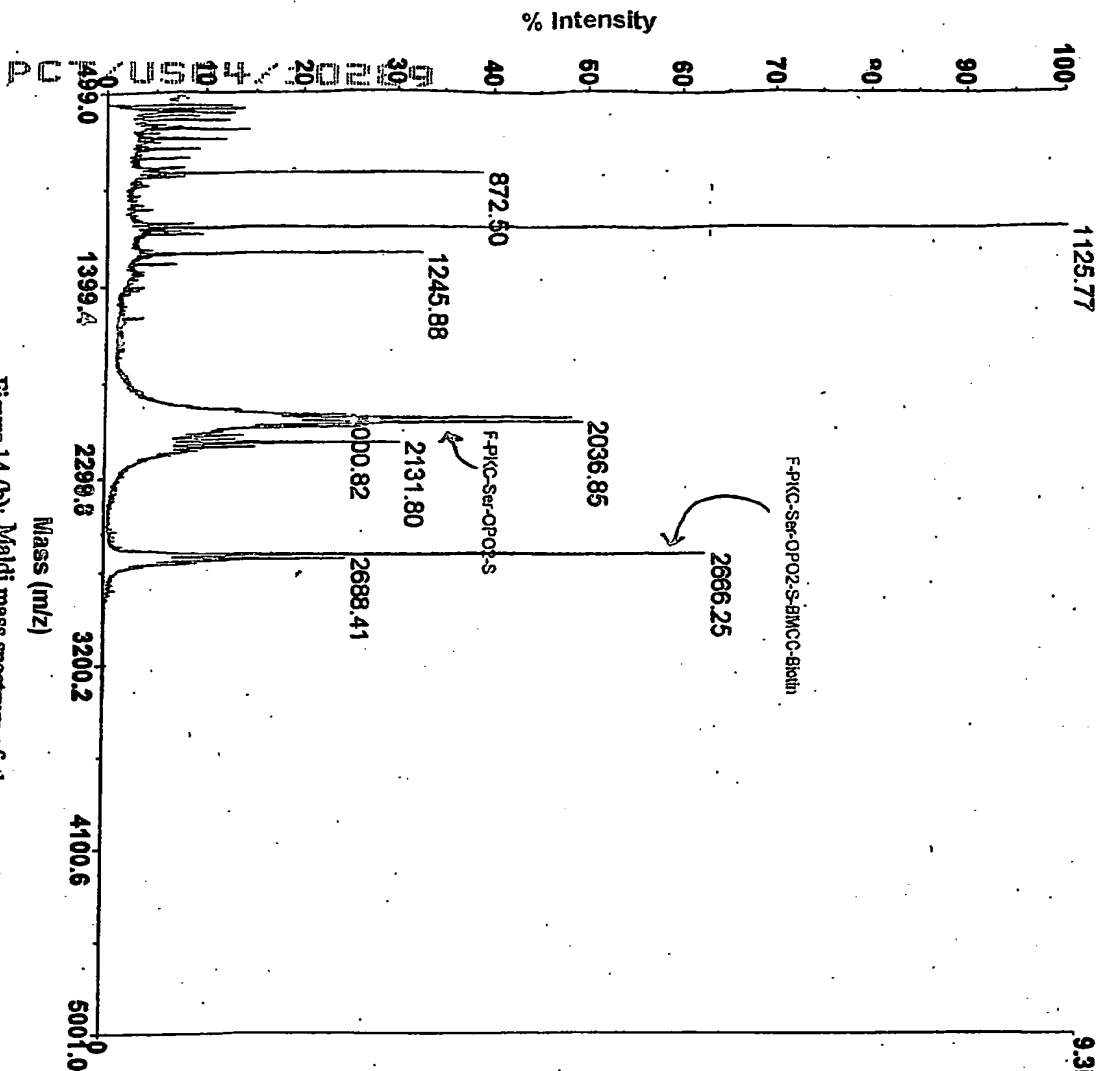
= Br-CH₂CONH--

= S

Figure 13. Protocol and chemistry of the present invention for phosphorothiolation and detection of fluoresceinated PKC-peptide target using the single step, nucleation effect rapid assay method and Chemistry III of the present invention

Applied Biosystems Voyager System 1197

Voyager Spec #1[BP = 1125.6, 9311]



Acquired: 17-02-00, September 21, 2001

Voyager data file: B559-11-B1_0002.dat

Figure 14 (b):

Maldi mass spectrum of the same phosphorothiolated, fluoresceinated PKC peptide target 15 minutes after addition of equimolar equivalents multiplexed Nucleation Centers preformed from avidin and the hetero-bifunctional Biotin-1197.

Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

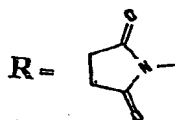
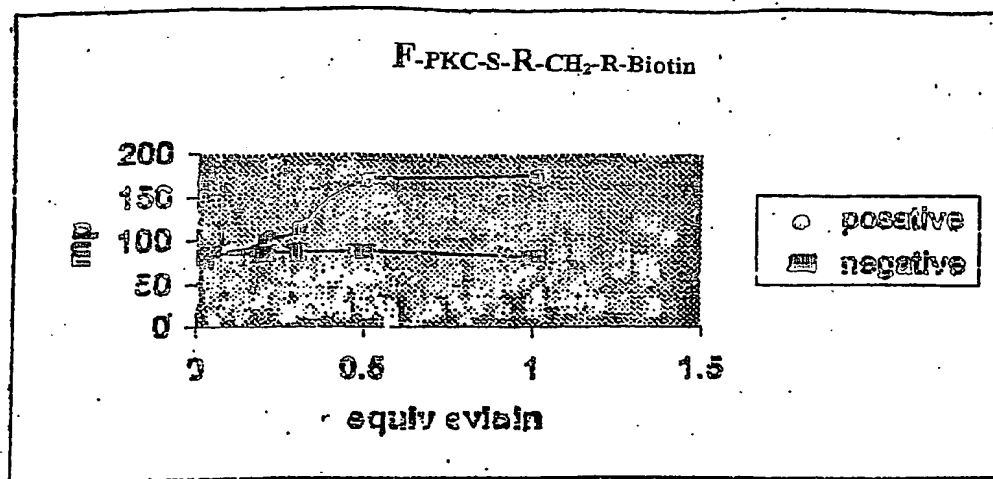
9.3E+3 Accelerating voltage: 20000 V
Grid voltage: 96%
Guide wire off: 0.05%
Extraction delay time: 200 msec

Acquisition mass range: 500 - 5000 Da
Number of laser shots: 100/spectrum
Laser intensity: 1640
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: B-Cyano-4-hydroxybenzoic acid
Low mass gate: 500 Da

Digitizer start time: 14.298
Bin size: 2 msec
Number of data points: 15324
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 47
Plate ID: RB109FEB21
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute x-position: 32051.3
Absolute y-position: 26104.1
Relative x-position: -16.1985
Relative y-position: -883.357
Shots in spectrum: 100
Source pressure: 9.673e-007
Mirror pressure: 0
TIC2 pressure: 0.01056
TIS gate width: 30
TIS flight length: 940

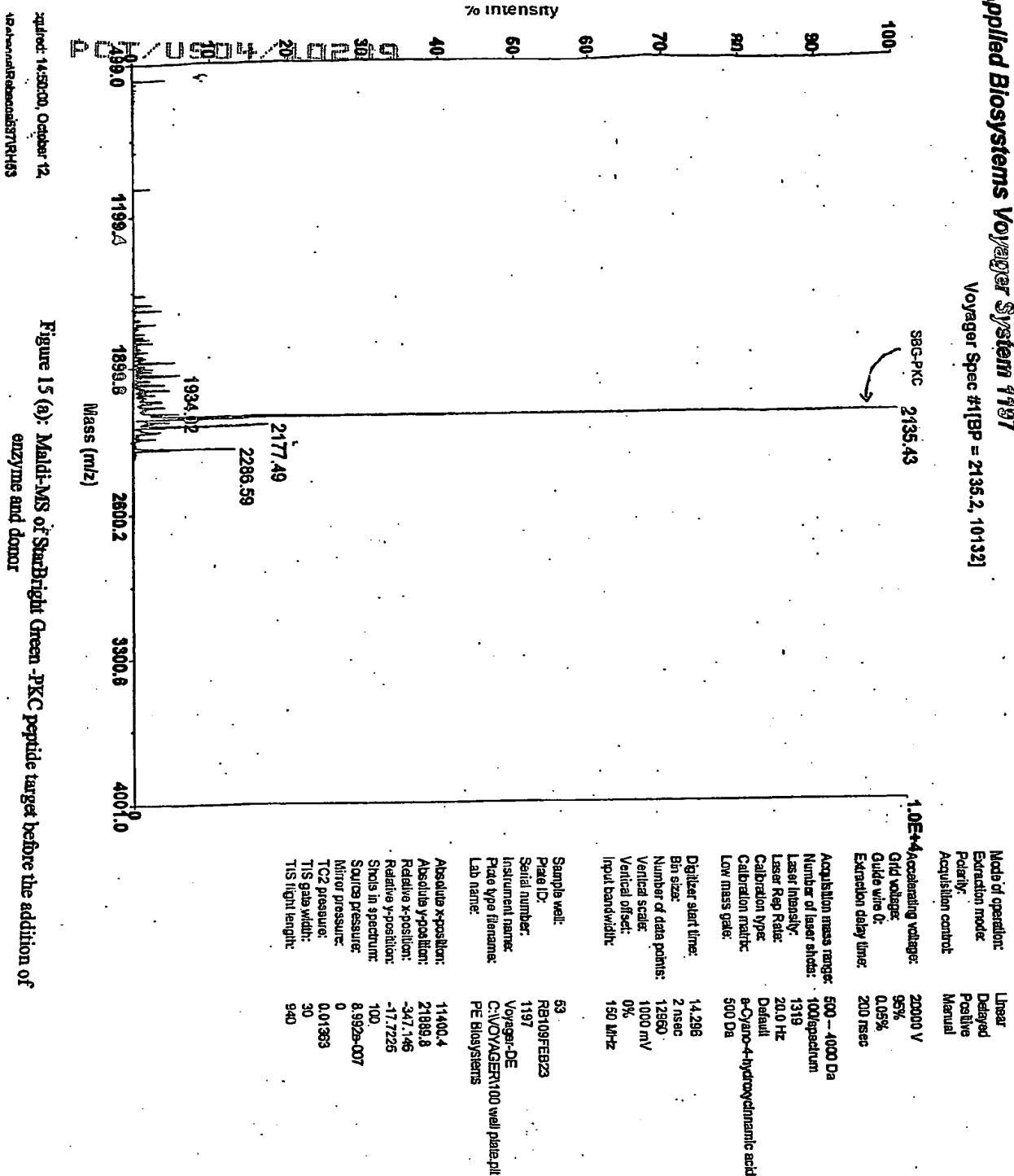


R = Long Chain alkyl

Figure 14 (C) Fluorescence polarization analysis of the same sample used to generate the spectrum of 14(b), above, showing the titration with multiplexed Nucleation Centers that were prepared from avidin and the hetero-bifunctional linkers, maleimido BMCC-biotin.

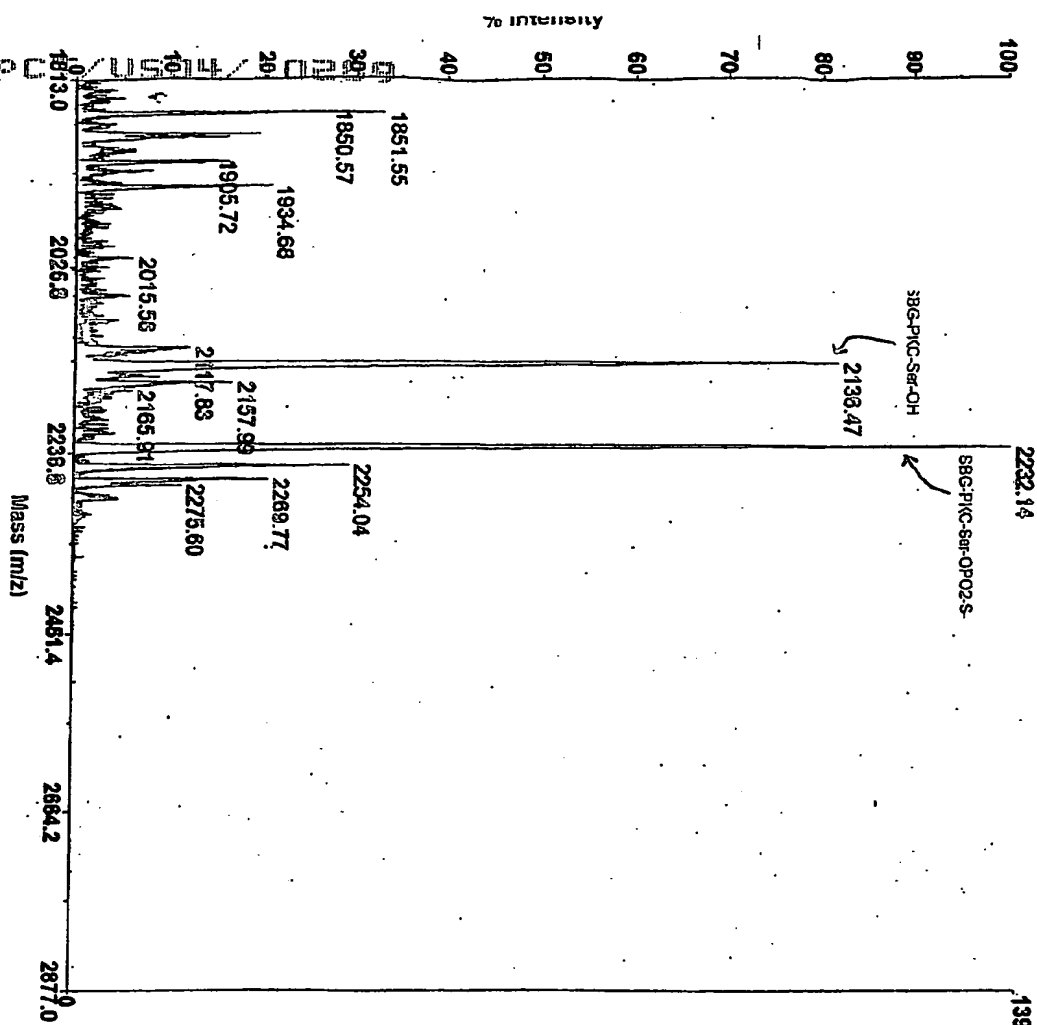
Applied Biosystems Voyager System 4197

Voyager Spec #1 [BP = 2135.2, 10132]



Applied Biosystems Voyager System 1197

Voyager Spec #1197 = 2231.9, 13981



Run: 12:12:00, October 11, 2001
 Reference: Reference 37 RH657-48Scan RT

Figure 15 (b): MS of Phosphorylated StarBright Green-PKC peptide target
 with enzyme and γ-S-ATP

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire 0: 0.05%
 Extraction delay time: 200 nsec

Acquisition mass range: 1000 - 4000 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1319
 Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: α-Cyano-4-hydroxycinnamic acid
 Low mass gate: 1000 Da

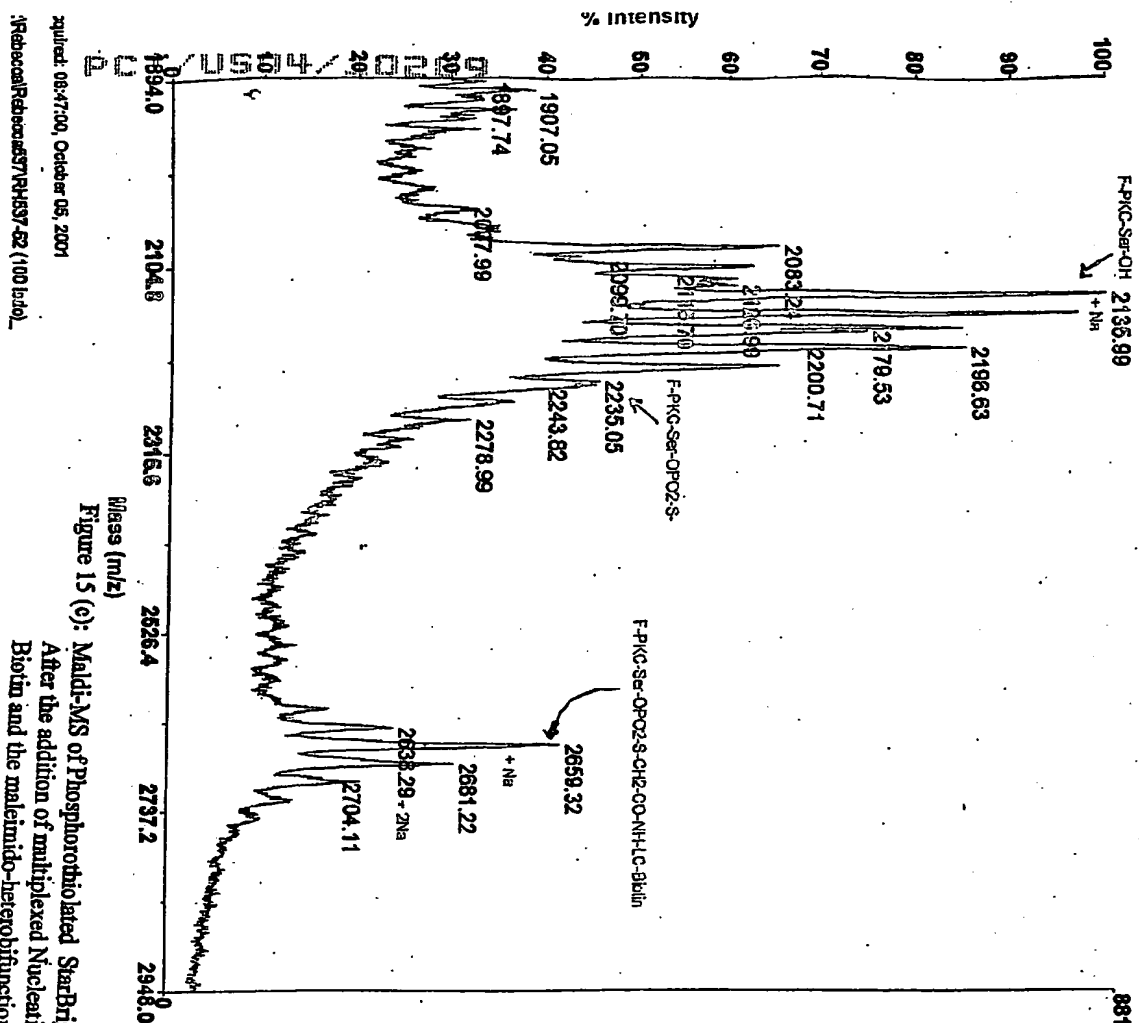
Digitizer start time: 20.17
 Bin size: 2 nsec
 Number of data points: 10023
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 24
 Plate ID: RB109FEB23
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.pl
 Lab name: PE Biosystems

Abscissa X-position: 18218.2
 Abscissa Y-position: 37102.9
 Relative X-position: -611.266
 Relative Y-position: -44.6175
 Shots in spectrum: 100
 Source pressure: 1.34e-006
 Minor pressure: 0
 TCC pressure: 0.07206
 TIS gate width: 30
 TIS flight length: 940

Applied Biosystems Voyager System 1197

F-PKC-Voyager Spec #1197 = 1894.6, 104831



Acq: 08:47:00, October 05, 2001
 Reference: Reference 57/1197-62 (100 to 100)

Figure 15 (c): MALDI-MS of Phosphorothiolated StarBright Green- PKC peptide target

After the addition of multiplexed Nucleation Centers preformed from Biotin and the maleimido-heterobifunctional linker

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual
 Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire off: 0.05%
 Extraction delay time: 200 nsec
 Acquisition mass range: 1000 - 3500 Da
 Number of laser shots: 1000 spectrum
 Laser intensity: 1747
 Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: a-Cyano-4-hydroxymethyl acid
 Low mass gate: 1000 Da
 Digitizer start time: 20.17
 Bin size: 2 nsec
 Number of data points: 8728
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz
 Sample well: 24
 Plate ID: RB108FEB23
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\1100 well plate.pit
 Lab name: PE Biosystems
 Absolute x-position: 17620.6
 Absolute y-position: 37815
 Relative x-position: 793.118
 Relative y-position: 667.546
 Shots in spectrum: 100
 Source pressure: 1.316e-008
 Mirror pressure: 0
 TCC pressure: 0.0714
 TIS gate width: 30
 TIS light length: 940

Fluorescence Polarization

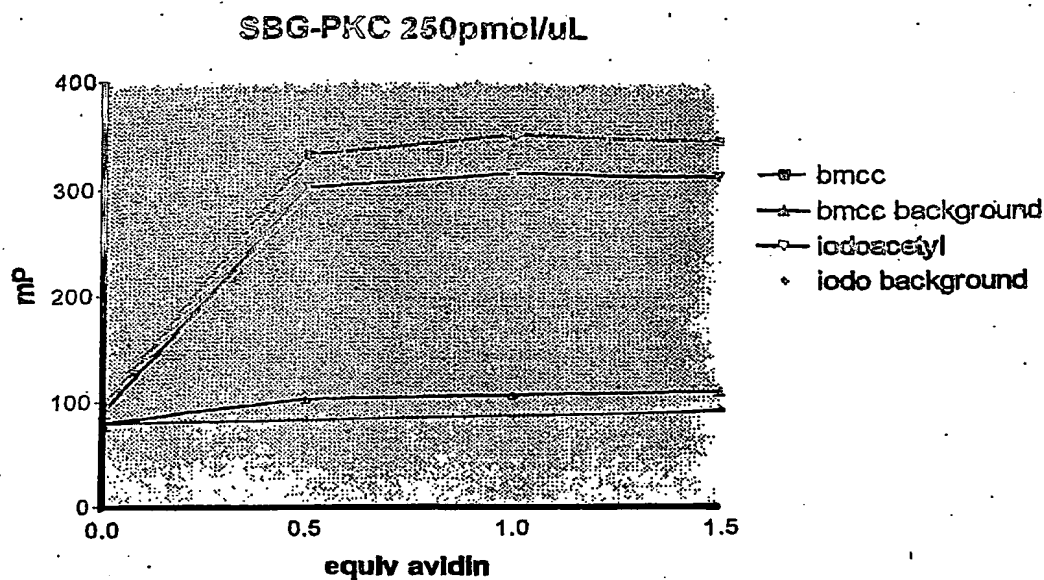
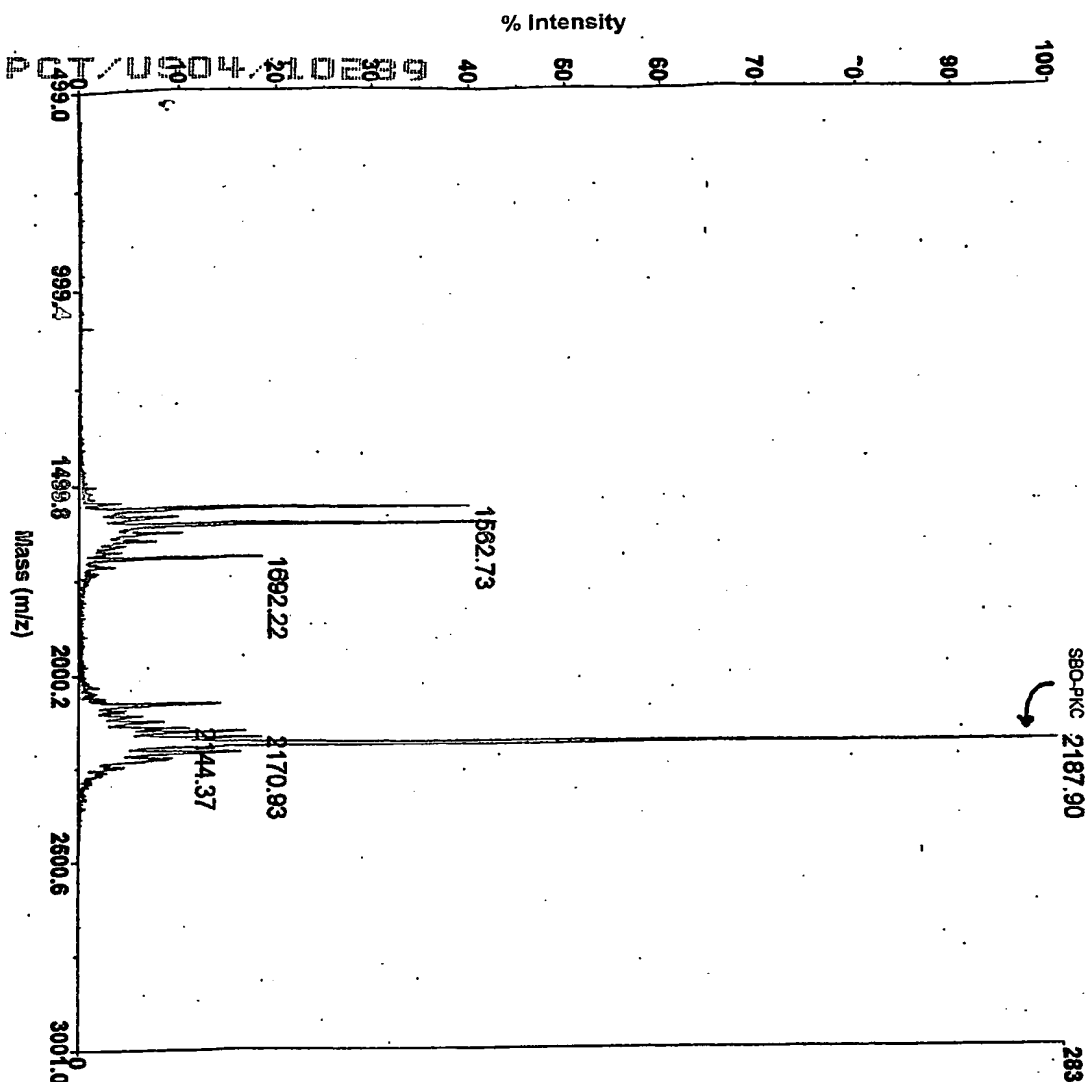


Figure 16. Fluorescence Polarization Analysis of the extent of the reaction of multiplexed Nucleation Centers prefomed from avidin and multiple heterobifunctional linkers bearing biotin at one terminus and maleimido- (blue line) and iodoacetamido- reactive groups at the other (purple line). The StarBright Green -PKC peptide target was phosphorylated by PKC-theta using γ -S-ATP as the donor.

Applied Biosystems Voyager System 1197

Voyager Spec #1BP = 2187.1, 28391



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire Ct: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 500 - 3000 Da
Number of laser shots: 100/spectrum
Laser intensity: 1141
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: a-Cyano-4-hydroxycinnamic acid
Low mass gate: 500 Da

Digitizer start time: 14.298
Bin size: 2 nsec
Number of data points: 10276
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 65
Plate ID: RB109FEB21
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute x-position: 22254
Absolute y-position: 16616.6
Relative x-position: 346.452
Relative y-position: -210.939
Shots in spectrum: 100
Source pressure: 8.339e-007
Mirror pressure: 0
TIC2 pressure: 0.01124
TIS gate width: 30
TIS flight length: 940

acquired: 16:44:00, August 22, 2001

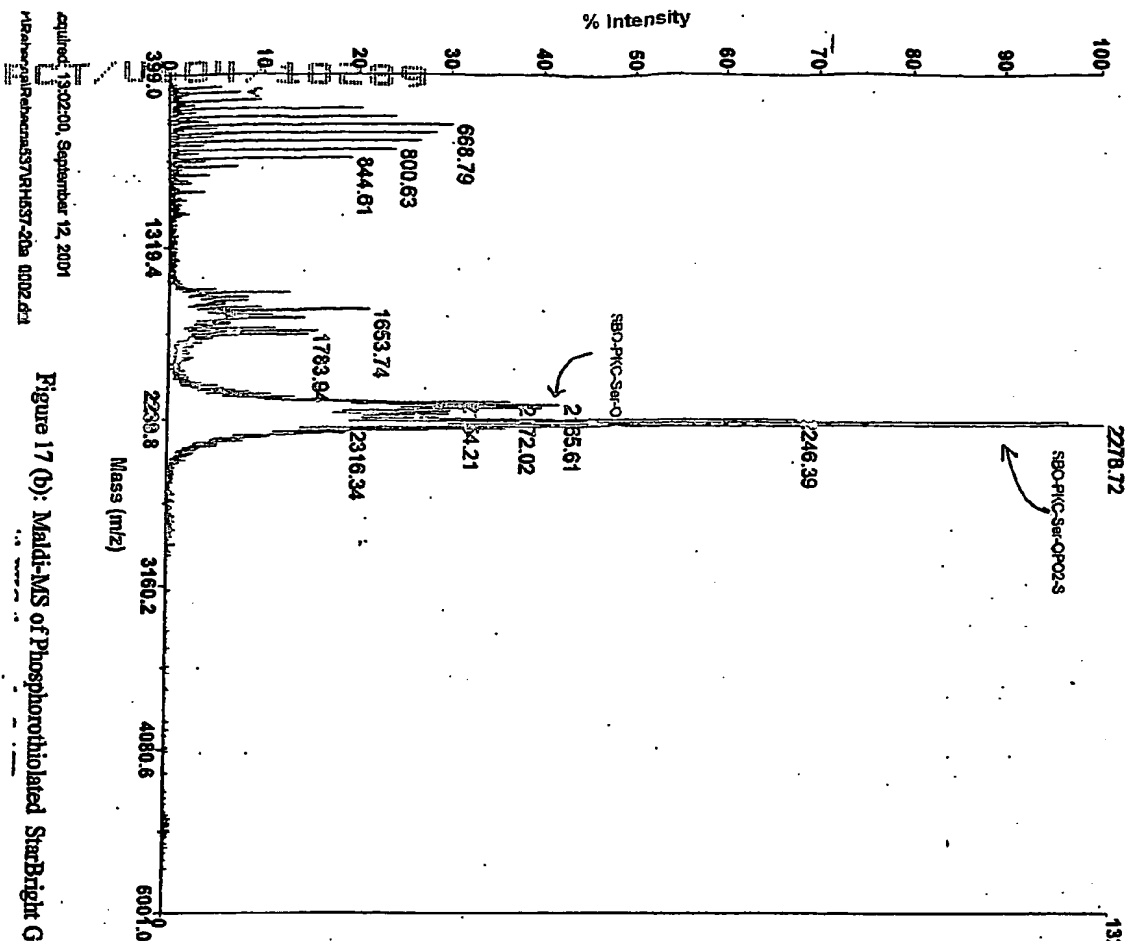
10/551507/10/551507-1 loaded 1

Figure 17 (a): MALDI-MS of StarBright Orange-PKC peptide target before the addition of enzyme and donor

arch 12, 2003

Applied Biosystems Voyager System 1197

Voyager Spec #1BP = 2277.8, 1326



Acquired: 13/02/200, September 12, 2001
 File Name: 13/02/2001-0912-01-0002-01

Figure 17 (b): MALDI-MS of Phosphorothiolated StarBright Green -PKC peptide target

Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	95%
Guide wire off:	0.05%
Extraction delay time:	200 msec
Acquisition mass range:	400 - 6000 Da
Number of laser shots:	1000/spectrum
Laser intensity:	1605
Laser Rep Rate:	20.0 Hz
Calibration type:	External - D-Voyagerdatacal2 sep4.cal
Calibration matrix:	a-Cyano-4-hydroxycinnamic acid
Low mass gate:	400 Da
Digitizer start time:	12.814
Bin size:	2.000
Number of data points:	16069
Vertical scale:	1000 mV
Vertical offset:	0%
Input bandwidth:	150 MHz
Sample well:	51
Plate ID:	RB109FEB21
Serial number:	1197
Instrument name:	Voyager-DE
Plate type filename:	C:\VOYAGER\1100 well plate.pl
Lab name:	PE Biosystems
Absolute x-position:	1166.12
Absolute y-position:	22267.5
Relative x-position:	-421.377
Relative y-position:	380.011
Shots in spectrum:	100
Source pressure:	3.391e-005
Mirror pressure:	0
TC2 pressure:	0.01269
TIS gate width:	30
TIS flight length:	940

Figure 18 (a) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-BMCC-LC-Biotin after the addition of Avidin

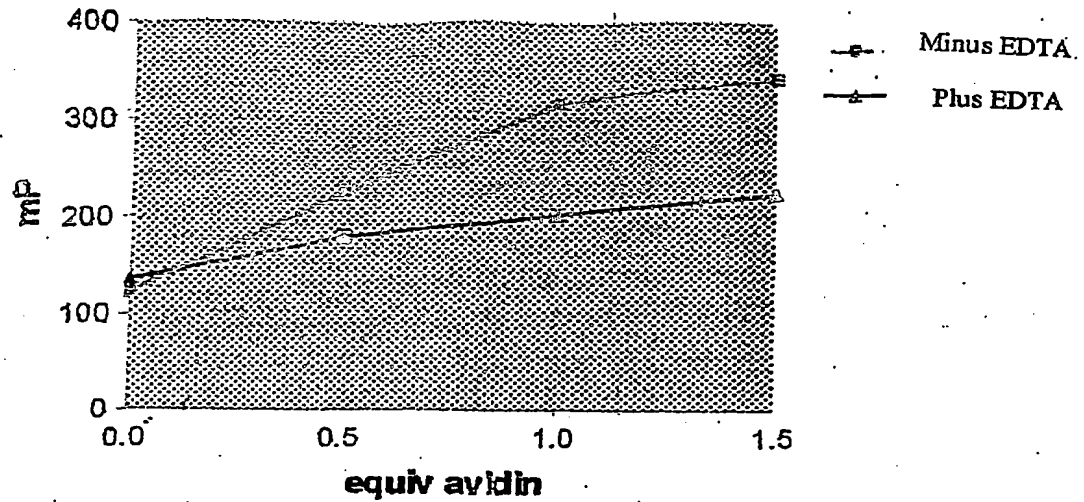
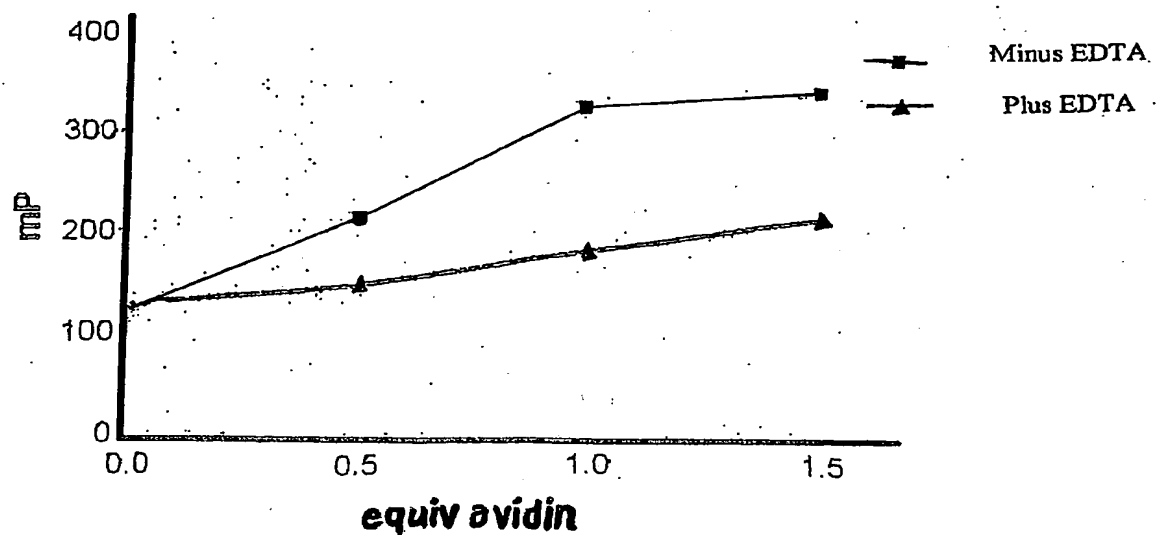
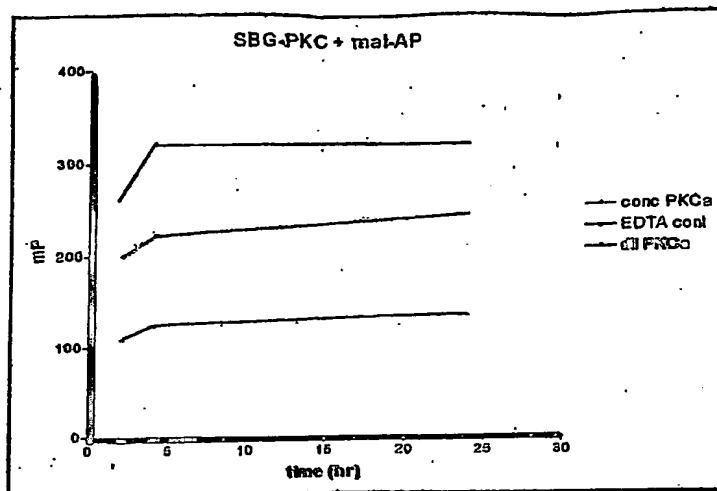


Figure 18 (b) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-Iodoacetyl-LC-Biotin after the addition of Avidine



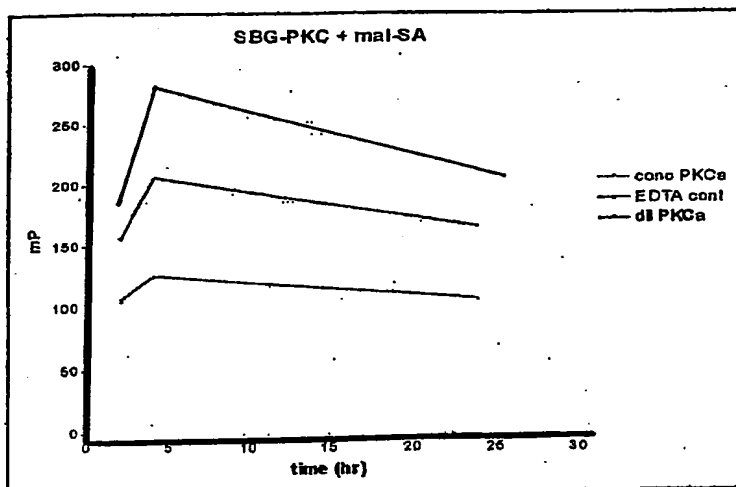
Fluorescence Polarization Using Large Molecules

(a)



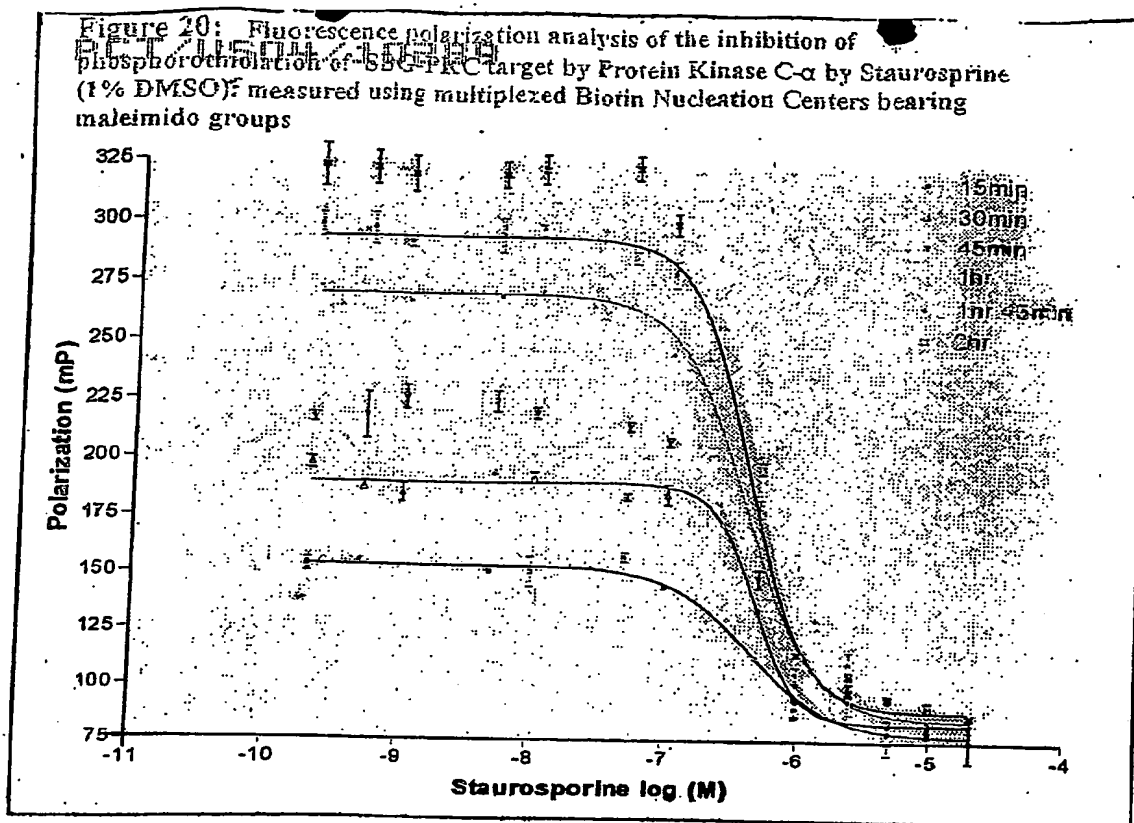
time (hr)	conc PKCa	EDTA cont	dil PKCa
2	267	110	200
4	321	125	223
24	321	136	245

(b)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	187	106	156
4	281	126	207
24	213	107	166

Figure 19: Fluorescence polarization of analysis of phosphorothiolated SBG-PKC after the addition of multiplexed Nucleation centers comprised of Alkaline Phosphatase, figure (a), and Streptavidine, figure (b), bearing multiple maleimido groups capable of reacting with the phosphorothiolated peptide described in figure 13.



EC50	4.39E-07	4.76E-07	4.40E-07	4.01E-07	4.16E-07	4.39E-07
KI	1.25E-07	1.36E-07	1.26E-07	1.15E-07	1.19E-07	1.26E-07

Fluorescence Polarization (mp)

15min		30min		45min		1hr		1hr45		2hr	
67	76	66	85	65	82	71	85	80	83	87	86
80	78	83	81	80	77	92	81	91	77	93	89
87	69	85	74	83	81	85	83	93	92	93	86
87	99	96	101	95	98	107	112	105	115	103	108
93	85	94	86	93	91	99	101	108	101	114	113
113	111	134	133	143	151	167	171	174	182	193	198
142	143	188	179	208	205	243	245	283	272	303	294
154	158	181	184	211	215	267	273	282	287	327	317
143	156	189	193	218	221	277	283	298	289	327	315
150	149	193	193	228	219	270	265	300	286	313	324
151	152	180	188	230	220	282	289	291	288	326	311
155	152	185	189	207	227	268	270	289	302	314	328
155	149	199	184	218	214	276	267	302	292	331	313
149	154	183	191	217	223	271	269	291	294	312	326
74	78	71	83	74	80	82	89	75	84	82	86
152	145	180	191	212	210	263	268	282	285	307	319

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